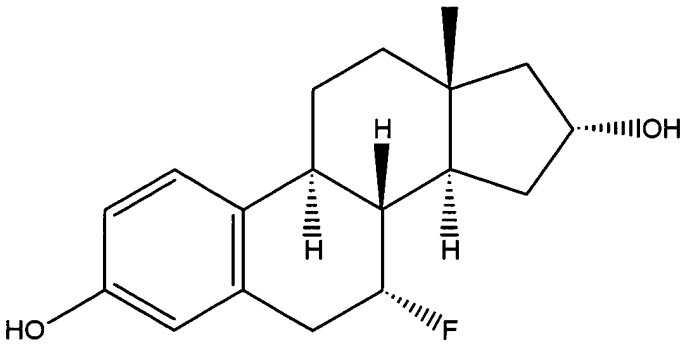

7-alpha-fluoro-estra-1,3,5(10)-triene-3,16alpha diol

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Thank you

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NEUTRAL C_{19}-STEROIDS AND STEROID SULFATES IN HUMAN PREGNANCY

II. DEHYDROEPIANDROSTERONE SULFATE, 16α-HYDROXYDEHYDROEPIANDROSTERONE, AND 16α-HYDROXYDEHYDROEPIANDROSTERONE SULFATE IN MATERNAL AND FETAL BLOOD OF PREGNANCIES WITH ANENCEPHALIC AND NORMAL FETUSES

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ABSTRACT

Dehydroepiandrosterone sulfate (DS), 16α-hydroxydehydroepiandrosterone sulfate (16α-OH-DS) and 16α-hydroxydehydroepiandrosterone (16α-OH-D) were measured in cord plasma of five anencephalic fetuses with histologically proven hypoplasia of the fetal zone of the adrenal cortex. DS was determined by the Zimmermann reaction on paper as described previously by us, the 16α-hydroxylated compounds by a modification of the method of Katz. These hormones were also measured in antecubital venous blood of the mothers of these fetuses. For comparison normal pregnant women and normal fetuses were studied. Neither 16α-OH-D nor 16α-OH-DS could be detected in peripheral blood of mothers bearing anencephalic or normal fetuses. In the former group, all DS levels (M±s = 93±31 μg%) were within the normal range. 16α-OH-D, 16α-OH-DS, or DS could not be detected in the cord plasma of anencephalic fetuses. If these steroids were present, their concentrations (< 10-25 μg DS%, < 9.5-36 μg 16α-OH-DS% and < 4 μg 16α-OH-D%) were markedly reduced as compared with normal fetuses for which values are given. Evidence is presented for the nature of 16α-OH-DS as a monosulfate, presumably the 3β-sulfate. The values reported for 16α-OH-DS are not corrected for losses; the corrected values would be about three times higher. It is hypothesized from this study that the decrease of 16α-OH-D, 16α-OH-DS, and DS in blood of anencephalic fetuses is due to the hypoplasia of the fetal zone of the adrenal cortex, and is the major cause of the low excretion of estrogens, especially estriol, in the maternal urine.

INTRODUCTION

In 1960 ten Berge reported an abnormally low value of urinary

estri 1 (estra-1,3,5(10)-triene-3,16α,17β-triol) in a woman pregnant with an anencephalic fetus (1). This was confirmed by Frandsen and Stakemann (2) in 1961, who also found estrone (3-hydroxy-estra-1,3,5(10)-trien-17-one) and estradiol (estra-1,3,5(10)-triene-3,17β-diol) to be reduced in 5 cases. Similar results were reported later by others (3, 4, 5, 6). Frandsen and Stakemann noted a correlation between the degree of hypoplasia of the fetal adrenal cortex and the amount of estrogens excreted by the mother (2). They put forth the hypothesis that the fetal adrenal cortex is involved in the elaboration of estrogens during pregnancy by producing either estrogenic hormones, or a stimulater for estrogen biosynthesis, or perhaps a precursor which is biotransformed to estrogens elsewhere. Based upon rat experiments with human placental and adrenal tissues Frandsen and Stakemann (7) later assumed that most likely the fetal adrenals produce a steroid precursor which is biotransformed into estrogens by the placenta. In a recent paper the same authors reported that dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one = D) injected intraamniotically results in an increase of urinary estrogens, predominantly in the estrone and estradiol fraction (8). Since estriol increased relatively little, Frandsen and Stakemann (8) concluded that the bulk of estriol excreted has other precursors.

In another approach, utilizing the measurement of estrogen and dehydroepiandrosterone sulfate (DS) production rates, MacDonald and Siiteri (9) found the production rate of estrone and estradiol to be reduced in patients pregnant with anencephalic fetuses. Parallel changes in DS-production were caused by the administration of either ACTH or dexamethasone. The authors calculated that the bulk of estrogen

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produced in these cases is derived from DS circulating in the maternal plasma. In addition, evidence was presented for the synthesis of estriol from DS through a pathway which does not involve estrone or estradiol. 16-hydroxylation of DS in the maternal compartment, (e.g. in the maternal liver) might be such a pathway (9).

If the hypothesis that the fetal adrenal neutral C_{19}-steroids are the major source of precursors of placental estrogens at term is correct, one would expect that the production of such precursors by the adrenals of anencephalic fetuses would be markedly reduced. These infants usually have a hypoplastic fetal zone of the adrenals which, indeed, does not synthesize cholesterol (10) or steroids from acetate in vitro (11).

Furthermore, one would expect C_{19}-steroids to be abnormally low in the blood of anencephalic fetuses. Results of measurements with older methods were controversial. Whereas DiGeorge, Arey and Bongiovanni (12) found high total neutral 17-ketosteroids in the plasma of an anencephalic fetus and later confirmed this finding in two other cases (13), Nichols, Lescure and Migeon (14) found no conjugated D in one case and very little in another. No free D could be detected by the latter authors. The discrepancy of these findings and the fact that the studies were done without control by internal standards, led us to restudy neutral C_{19}-steroids and their sulfates in the cord plasma of anencephalic fetuses.

In 1964 we reported markedly decreased concentrations of DS, corrected on the basis of recovered 4-^{14}C-DS, in the cord plasma of two anencephalic babies (15). More recently, Colás and Heinrichs (6)

published measurements of uncorrected subnormal amounts of total D and total 16α-hydroxydehydroepiandrosterone (3β,16α-dihydroxy-androst-5-en-17-one = 16α-OH-D) in the plasma of four anencephalic fetuses. Furthermore, Eberlein, found very little total Δ^5-3β-hydroxysteroid sulfates in the blood of a three day old anencephalic fetus before and after ACTH administration (16).

As to the levels of D in the maternal peripheral blood of such cases, again controversial results have been reported. Whereas Nichols et al. (14) found a markedly decreased value for conjugated D in one case, Colás and Heinrichs (6) measured normal values of total D in three out of four cases. Only one case had a slightly decreased value.

We are now presenting a more extended report of our findings on DS, and on "free" 16α-OH-D and 16α-OH-DS in five anencephalic fetuses with histologically proven hypoplasia of the fetal adrenal cortical zone. Values for these steroids in peripheral blood of the five mothers will also be reported. Normal values are included in all instances for comparison. Finally, we will present evidence as to the identity of 16α-OH-DS in cord plasma as a monosulfate.

MATERIALS AND METHODS

Five anencephalic fetuses and their mothers were studied. Clinical data on them is summarized in table I. All patients had polyhydramnios. Autopsies and histological examinations of the adrenals were performed on all fetuses. Fetal pituitaries were not recognizable in cases I and V. Of the remaining cases only the pituitary of case III was studied microscopically. The histology of this pituitary appeared to be normal (17).

For comparison plasma samples of normal mothers with normal fetuses were studied. The results for dehydroepiandrosterone sulfate in 20 normal cases have been reported previously (15). In addition, plasma pools of 16 normal mothers and their fetuses were analyzed for 16α-OH-DS.

TABLE I

CLINICAL DATA ON FIVE ANENCEPHALIC FETUSES STUDIED

CASE	PREGNANCY			INFANT				ADRENALS	
	MOTHER AGE	DURATION (WEEKS)	METHOD OF DELIVERY	SEX	WEIGHT g	LENGTH cm	DURATION OF LIFE	TOTAL WEIGHT	FETAL ZONE
I C.G.	27	38	vaginal	female	2600	42	1 min.	2 g	hypoplasia
II T.P.	30	35	vaginal	female	1360	36	1 min.	unknown	hypoplasia
III B.R.	23	39	cesarian section (repeat)	female	1830	46	2 min.	2 g	hypoplasia
IV B.D.	23	36	vaginal	male	2000	47	died during delivery	1.2 g	hypoplasia
V C.K.	19	40	vaginal	female	2140	?	8 min.	1 g	hypoplasia

The volume of the p ls varied between 10 and 15 ml, and always contained either equal amounts of fetal arterial, fetal venous or maternal peripheral blood from a given case. Larger pools were analyzed for 16α-OH-D (see table IV). Techniques for the collection of blood and preparation of the plasma were as described previously (15).

Dehydroepiandrosterone sulfate (DS) was determined and corrected for losses as described in an earlier paper (15).

Unconjugated 16α-hydroxydehydroepiandrosterone (16α-OH-D)

a. Quantitative measurement The so-called free steroids were extracted, purified and chromatographed according to the method of Finkelstein, Forchielli, and Dorfman (18). The zone corresponding to authentic 16α-OH-D on the first paper chromatogram was rechromatographed on Whatman #2 paper with toluol:n-heptane:methanol:water (500:500:300:150) at 29° C.

16α-OH-D was detected on paper by a modification of the method of Katz (19) (AACS test). Chloroform was mixed with antimony trichloride (Mallinckrodt) on a magnetic plate until only a few crystals remained. The saturated solution was centrifuged and the clear supernatant was transferred to a separatory funnel before the addition of 1 ml of anisaldehyde and 2 ml of reagent grade sulfuric acid. After shaking one minute the phases were allowed to separate in the dark for one and a half hours. The paper was dipped in the chloroform solution two times, air dried and heated in an oven at 90° C for six minutes. This procedure gave a more intense and constant slate blue color with 16α-OH-D than did the original method.

The quantification was performed as with DS (15) using the Spinco Analytrol according to Conrad and coworkers (20). The limit of detection was approximately 1 μg of 16α-OH-D distributed over a 2 cm^2 area.

The recovery was determined by the addition of 7α-^{3}H-16α-OH-D (21) to plasma and scanning the paper chromatograms before colorimetry with a 4pi-radioactograph (Nuclear Chicago) and then measuring the areas under the peaks as recommended by Berliner et al.(22).

b. Specifity By the chromatography and the color test applied none of the steroids discovered thus far in cord plasma (6, 16) were found to interfere with the measurement of 16α-OH-D. Specifically, in the paper chromatography system employed, the following di- and trihydroxysteroids and monohydroxy-dioxosteroids (23) were separable from 16α-OH-D:

3β,17β-dihydroxy-androst-5-en-16-one	androst-4-ene-3β,17β-diol
16α,17β-dihydroxy-androst-4-en-3-one	pregn-5-ene-3β,20α-diol
androst-5-ene-3β,17β-diol	5β-pregnane-3α,20α-diol
androst-5-ene-3β,16α,17β-triol	pregn-5-ene-3β,20β-diol
3β,16α-dihydroxy-pregn-5-en-20-one	17β-hydroxy-pregn-4-en-3,20-dione

It must be said, however, that our method provides only tentative evidence for the presence of unconjugated 16α-OH-D in cord plasma as does the method used by Magendantz and Ryan (25, 26).

16α-hydroxydehydroepiandrosterone sulfate (16α-OH-DS)

a. Quantitative measurement After extraction of unconjugated steroids

the sulfate was extracted and paper chromatographed together with DS as described earlier (15). The 16α-OH-DS zone was eluted and rechromatographed in butyl ether:n-butanol:ammonium hydroxide:water (500:500:100:900) on Whatman #2 paper at 29° C. 16α-OH-DS was then eluted and solvolyzed according to the method of Burstein and Lieberman (26). The liberated 16α-OH-D was chromatographed on Whatman #2 paper with toluol:n-heptane:methanol:water (500:500:300:150) at 29° C, colored and quantitated with the AACS reagent as described above. In three instances tested, the recovery of 7α-^{3}H-16α-OH-DS indicated an accuracy of about 30%. The values reported are uncorrected.

b. Evidence for the presence of a monosulfate

1. Extractability as the pyridinium salt with chloroform (27).

2. Paper chromatography in three Schneider and Lewbart (28) systems: a) butyl ether:tert.-butanol:ammonium hydroxide:water (500:500:100:900), b) butyl ether:n-butanol:ammonium hydroxide:water (500:500:100:900), c) toluene:ethyl acetate:n-butanol:ammonium hydroxide:water (400:100:500:100:900); column chromatography on Florisil (20); thin layer chromatography on silica gel in two different systems: water saturated butanol, and water saturated benzene:methanol (50:50).

The substance under discussion was detected in the monosulfate area on paper, and on thin layer, and in the monosulfate fraction eluted from the column, respectively. It had the same chromatographic properties as a monosulfate of authentic 7α-^{3}H-16α-OH-D prepared by the method of Sobel and Spoerri (29).

3. The following tests were positive on paper: rhodizonic acid test (30), phosphomolybdic acid test (31), AACS test (19), blue tetrazolium test (32); the Zimmermann test on paper (33) was negative. The methylen blue (34) and Pettenkofer test (35) were positive in solution.

4. The failure of precipitation with digitonin as judged by thin layer chromatography on silica gel using water saturated butanol in which system no splitting of the dehydroepiandrosterone digitonide occurs. This could indicate that the compound isolated from cord plasma is esterified with sulfuric acid in position 3β. However, it should be pointed out that substitutions on the D-ring are known to interfere with digitonide formation.

Other thin layer and paper chromatography systems were tried first and discarded because the digitonide was unstable in them.

5. Acetylation with 1-^{14}C-acetic anhydride and pyridine (3:1).

6. Solvolysis with sulfuric acid in ethyl acetate (26). Isolation of 16α-OH-D by two Bush systems and detection by the AACS test as described above.

RESULTS

Our results for dehydroepiandrosterone sulfate (DS) are summarized in table II. In the cord plasma of anencephalic fetuses no DS could be detected. The different limits of detection are due to differences in plasma volume. If DS is present, it's concentration is below the range

TABLE II

DEHYDROEPIANDROSTERONE SULFATE (DS) IN PLASMA OF ANTECUBITAL MATERNAL VENOUS BLOOD AND CORD BLOOD IN PREGNANCIES WITH ANENCEPHALIC FETUSES. COMPARISON WITH NORMAL FETUSES AND MOTHERS.

PATIENTS	µg % PLASMA* MIXED CORD BLOOD	MATERNAL ANTECUBITAL VENOUS BLOOD
I	< 10	104
II	< 10	51
III	< 20	117
IV	< 25	122
V	< 15** < 10***	70
normal fetuses cord vein n = 20	M = 130 range = 38-252 s = ± 59	-
normal fetuses cord artery n = 20	M = 162 range = 82-306 s = ± 62	-
normal mothers peripheral vein n = 20	-	M = 100 range = 35-257 s = ± 52

*corrected for losses **cord arterial blood ***cord venous blood

observed in cord venous and arterial blood of normal fetuses (15). On the other hand, all values in the maternal antecubital venous blood (M±s = 93±31 µg%) were within the range of normal pregnant women at delivery. Only one value was in the low normal range.

16α-hydroxydehydroepiandrosterone sulfate (16α-OH-DS) was not detected in any of the maternal samples, as indicated in table III. Neither was it detected in the four anencephalic fetuses studied, however it was measurable in all pools of cord arterial and venous blood of nor-

TABLE III

16α-HYDROXYDEHYDROEPIANDROSTERONE SULFATE (16α-OH-DS) IN CORD PLASMA OF ANENCEPHALIC FETUSES AND IN PERIPHERAL PLASMA OF THEIR MOTHERS. COMPARISON WITH NORMAL FETUSES AND NORMAL MOTHERS.

PATIENTS	μg % PLASMA* MIXED CORD BLOOD	μg % PLASMA* MATERNAL ANTECUBITAL VENOUS BLOOD
I	-	-
II	< 10	< 10
III	< 9	< 9
IV	< 36	< 30
V	< 18** < 20***	< 20
normal fetuses cord vein 6 pools from 16 samples	M = 87 range = 31-148	-
normal fetuses cord artery 6 pools from 16 samples	M = 110 range = 35-250	-
normal mothers peripheral vein 6 pools from 16 samples	-	< 10

*uncorrected for losses **cord artery ***cord vein

mal fetuses. On the average, we found 87 μg% of 16α-OH-DS in cord venous blood and 110 μg% in cord arterial blood. Although the mean values show an arteriovenous difference similar to that found for DS (15) no statement can be made about individual differences since 16α-OH-DS measurements were performed on pools of plasma. The values were not corrected for losses. The real values are probably three times higher (see methods).

TABLE IV

"FREE" 16α-HYDROXYDEHYDROEPIANDROSTERONE (16α-OH-D) IN POOLS OF CORD PLASMA OF ANENCEPHALIC FETUSES AND IN POOLS OF PERIPHERAL PLASMA OF THEIR MOTHERS. COMPARISON WITH NORMAL MOTHERS AND THEIR FETUSES.

PATIENTS	PLASMA OF CORD POOLS Vol. in ml	μg %*	PLASMA OF MATERNAL POOLS Vol. in ml	μg %*
No. I-V	53	< 4	46	< 10
12 normal fetuses cord vein	56	17	-	-
12 normal fetuses cord artery	47	24	-	-
12 normal mothers peripheral vein	-	-	57	< 8

*corrected for losses

As shown in table IV, "free" 16α-hydroxydehydroepiandrosterone (16α-OH-D) was not found in a pool of plasma obtained from normal mothers at delivery nor in the pooled plasma of the five mothers bearing anencephalic fetuses. No 16α-OH-D was detected in the plasma of these fetuses. If this plasma contained 16α-OH-D it was less than 4 μg%. On the other hand, the pools of cord venous and arterial blood of normal fetuses contained 17 μg% and 24.5 μg% 16α-OH-D respectively, These values are corrected for losses. Again, although there seems to be an arteriovenous difference, no statement can be made about the differences in individual pairs since analyses were made on plasma pools.

DISCUSSION

To our observations on dehydroepiandrosterone sulfate (DS) in the cord plasma of two anencephalic fetuses reported in 1964 (15), we are adding three additional cases. Since all analyses were performed with 4-^{14}C-DS as internal standard we can be definite that very little, if any DS is circulating in the fetal blood of anencephalics. Thus, the findings on conjugated D by Nichols et al. (14) and total D by Colás and Heinrichs (6) can be now interpreted as being due to a large decrease in DS.

It is more difficult to explain the high values of total 17-ketosteroids in the cord plasma of anencephalic fetuses with marked hypoplasia of the adrenal fetal zone as observed by DiGeorge et al. (12, 13). We did not detect any 17-ketosteroid sulfates in the cord blood of our anencephalic fetuses. The analyses of Nichols et al. (14) provided no evidence for such conjugates and furthermore, these authors did not detect any 17-ketosteroids in the glucuronoside fraction. However, they did present evidence for a Δ^4-3-ketosteroid. In addition, androsterone and progesterone were tentatively identified as free steroids, the former in small amounts, the latter in larger concentrations. Since 3- and 20-ketosteroids can cause falsely high values for 17-ketosteroids as measured by the Zimmermann reaction (36), the high values reported by DiGeorge et al. (12, 13) might have been at least partly due to such steroids, presumably progesterone (14) and its metabolites. This view is further supported by the finding of a normal excretion of pregnandiol in pregnant women with anencephalic fetuses (2, 3, 5, 6, 8, 35) and the

detection of various C_{21}-steroids and steroid-conjugates after injection of isotopic progesterone into an anencephalic fetus (37). Δ^5-3β-hydroxysteroids, e.g. 17α-hydroxypregnenolone, have also been reported in anencephaly, although their levels in cord plasma were reduced (16). Thus far, however, none of the C_{21}-steroids mentioned has been sufficiently identified in the blood of anencephalic fetuses. Finally, nonspecific chromogens could have contributed to the high "17-ketosteroid" values in these cases (38).

A marked reduction of circulating total 16α-hydroxydehydroepiandrosterone (16α-OH-D) in anencephalic fetuses has been reported by Colás and Heinrichs (6). We confirmed their results and were able to show that the reduction is due to a decrease in unconjugated 16α-OH-D and its sulfate. We feel that we obtained good evidence that this sulfate is a monosulfate. The failure of precipitation with digitonin could indicate that 16α-OH-D is sulfo-conjugated in position 3β. A full identification, however, is still needed.

As to the levels of 16α-OH-D and conjugated 16α-OH-D in normal cord plasma reported by others (24, 25, 35) and by the authors the following can be pointed out: As to the amounts of tentatively identified 16α-OH-D there is good agreement between our findings and those of Magendantz and Ryan (25). Our values for 16α-OH-DS are lower than their postsolvolysis values. The procedures differ and therefore probably have different accuracies. No corrections for losses were made in either study. Furthermore, our values were calculated as 16α-OH-DS, whereas Magendantz and Ryan expressed their values in terms of the free steroid. In three instances we determined the recovery of 7α-^{3}H-16α-OH-

DS and found it to be about 30% in our procedure. This would suggest that the true mean value for cord arterial plasma is about 360 µg%. The value for total 16α-OH-D in cord arterial plasma reported by Colás et al. (35) would be even higher if corrections were introduced for the color difference between 16α-OH-D and D employed as reference standard, and furthermore for the difference between 16α-OH-D and 16α-OH-DS, and finally for losses. It is not surprising that such a value would be even higher than our calculated one if one considers that Colás et al. (35) measured conjugated and unconjugated 16α-OH-D together and that 3β, 17β-dihydroxyandrost-5-en-16-one was as a minor contaminant in their 16α-OH-D fraction (6). In any case, such calculations and comparisons furthermore indicate that the monosulfate, presumably the 3β-sulfate, is the major form in which 16α-OH-D circulates in the fetus.

Although the figures presented in this paper seem to indicate that DS is the major C_{19}-steroid sulfate in cord plasma, 16α-OH-DS, when corrected for losses, is certainly the C_{19}-steroid sulfate with the highest concentration. The data of Colás et al. (35) regarding total D and total 16α-OH-D are a reflection of this finding. The very low levels for esterified 16α-OH-D reported by Eberlein (16) are, according to the author, probably due to losses in the procedure used in that study.

Surprising is the broad normal range of total or conjugated 16α-OH-D as observed by Colás et al. (35), Magendantz and Ryan (25) and the authors. We previously discussed a fluctuation of the fetal blood flow and the adrenal function as possible causes (15) for the similarly large normal range for DS. Two observations are in favor of the latter cause: the occasional occurrence of degenerative changes of the fetal

adrenal zone already before birth (39), and the tremendeous variation of the ACTH concentration in cord plasma to be discussed later.

In anencephalic fetuses DS, 16-OH-DS and 16α-OH-D in cord blood were so markedly reduced that they could not be measured by the methods used in this study. All fetuses in our series died after a few minutes, one expired shortly before delivery. It might be pointed out that in all cases (except the one death) there was no sign of fetal distress during labor up to the moment of delivery. If we assume that the half life of DS in fetuses is similar to that in adults, e.g. in the order of several hours, it is difficult to accept that the sudden death at delivery or shortly thereafter could have significantly changed the concentrations of 16α-OH-DS and DS. In addition it might be mentioned that Colás and Heinrichs (6) also found extremely low values for total D and 16α-OH-D in an anencephalic baby who expired four hours after birth. Although impending death cannot be ruled out as a factor involved in the lowering of circulating steroids in the anencephalic fetus we share the hypothesis that the marked hypoplasia of the fetal adrenal cortex is the major if not the only cause of the reduction in circulating C_{19}-steroids. Nichols et al. (14) were the first ones to express this view. The failure of the hypoplastic adrenal of anencephalics to form cholesterol (10) or steroids (11) from acetate supports this view as do the studies of the 3β-hydroxysteroid dehydrogenase and steroid 3β-sulfatase in the tissues of two anencephalic fetuses by Goldman et al. (40). These studies suggest that the decreased values of DS could not be due to an unusually high rate of removal of circulating DS by tissue metabolism (40). However, one must consider the role of the placenta of anenceph-

alic fetuses with an apparently higher sulfatase activity (41).

Whether or not the normal fetal adrenal cortex secretes D, 16α-OH-D, DS, and 16α-OH-DS is not established. Formation of D from acetate with whole adrenals (42, 43) and fetal zones (11) as well as formation of DS from pregnenolone (3β-hydroxy-pregn-5-en-20-one) (44) has been shown in vitro. Although 16α-hydroxylation occurred with other steroids in such experiments (43, 45, 46) 16α-OH-D and 16α-OH-DS have not been detected. Adrenal secretion of none of these steroids has been demonstrated, for obvious reasons. On the other hand, it has been shown that the fetal liver is capable of 16α-hydroxylation of D in the rat (47, 48) and in the human (49). In particular, liver microsomes of anencephalic infants were very active in 16α-hydroxylating D (48). The marked reduction of estriol with an increase of estrone and estradiol-17β in the urine of a pregnant woman bearing a fetus with liver cirrhosis indirectly supports the concept that the human liver is, also in vivo, at least participating in 16α-hydroxylation. In addition, the very rapid sulfoconjugation of estriol (50) and of progesterone (37) injected into anencephalic fetuses suggests that organs other than the adrenals, most likely the liver, sulfoconjugate steroids. Although there is no doubt concerning extra-adrenal 16α-hydroxylation and sulfoconjugation of D in the human fetus, the extent of these reactions cannot be assessed at the present time.

The etiology of anencephaly is unknown (52, 53). The findings to date suggest a complex influence of genetic and exogenous factors (53). As early as 1912, Meyer proposed that the partial or complete lack of the fetal zone of the adrenal cortex was caused by the anencephaly (54).

He also stated that the mechanism of this relationship was unknown. Browne (55), in 1920, related the adrenal hypoplasia to the apituitarism of these infants. Others, however, found an anterior lobe of the pituitary, more or less intact, but no posterior lobe (56, 57) was present. Angevine (57) in 1938, considered the adrenal hypoplasia to be due to the impaired development of the pituitary, "induced by changes in the tissues at the site of the base of the brain".

Since the exact mechanism by which the normal adrenal becomes hypertrophic is not fully understood (16, 58), the mechanism by which the fetal zone is reduced in anencephalic fetuses is also not known. As early as 1931 the development of the fetal zone was considered to be a specific reaction of the fetus to pregnancy (59, 60). More specifically Rotter, in 1949, claimed that HCG is the fetal adrenal stimulus (61). Fetal pituitary LH has also been thought to be the stimulating hormone of the fetal adrenal (62). In 1955, Jones reemphasized the hypothesis that HCG is the adrenal stimulus (63), and Lauritzen and Lehmann (58) recently presented new experimental evidence in support of this hypothesis. They observed an increase of conjugated D in urine of newborn babies in response to HCG. However, this might be explained by an increase in gonadal function.

In any case, increasing evidence points to fetal ACTH as the stimulus for the fetal adrenal gland. In rats, this is well established by Jost (64). In the human, thus far, evidence is more circumstantial. In the experiments of Lanman HCG had no influence upon the hypoplastic adrenal cortex of anencephalic fetuses who survived for a few days, whereas the use of long acting ACTH resulted in the development of nor-

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mal sized adrenals in one case (65). In more recent similar studies, Lanman, in five cases achieved adrenals consistent in size and morphology with normal newborn glands (66). Steroid studies in one anencephalic fetus treated from the third to the fifth day with ACTH did not reveal any response (67), but the time of treatment might have been too short.

The lack of ACTH in cord plasma of the anencephalic fetuses, which we observed in one case (68), would provide further evidence for the role of ACTH if the normal range of ACTH in cord blood were known. This range is very large. With the bioassays used however, the lower normal range could not be determined (68, 69). Therefore it cannot be decided whether or not anencephalic fetuses have reduced ACTH levels in blood. Interestingly, considerable amounts of ACTH could be detected in three pituitaries of anencephalics studied thus far (68, 70). Whether the concentrations found are normal or not cannot be said, since data on ACTH concentrations in pituitaries of normal fetuses (68, 71, 72, 73) is insufficient. In any case, ACTH is produced by these pituitaries, but apparently not released, most likely due to a lack of ACTH releasing factor (CRF), as postulated by Schwers (70).

Additional evidence for fetal pituitary ACTH as the stimulating hormone for the fetal adrenal gland stems from observations obtained after administration of corticoids to pregnant women before and during delivery. This leads to a decrease of C_{19}-steroid sulfates in cord plasma, presumably via suppression of ACTH release by the fetal pituitary. We will report these observations on six cases in a subsequent paper (74).

Thus, available data favors ACTH, and not HCG as the fetal adrenal cortex stimulating hormone at term, and its lack in anencephalic fetuses.

Finally, the decreased excretion of estrogens in the urine of mothers bearing anencephalic fetuses (1, 2, 3, 4, 5, 6) can now be explained by a large decrease of at least three fetal precursors of placental estrogens: DS, 16α-OH-DS and 16α-OH-D. The experimental evidence for the role of these steroids as precursors of estrogens has been reviewed recently by several authors (9, 15, 67, 75).

Another mechanism, involving the impairment of the placenta of anencephalic fetuses should be considered. According to ten Berge the epithelium of the placental villi is severely damaged, and the stroma is also abnormal (76). Ten Berge claims that these tissue alterations decrease the capillary action of the villous vessels, thus reducing the hormone transport from placenta to mother. This results in a decreased excretion of estrogens, especially estriol (76).

MacDonald and Siiteri (9) have demonstrated that the bulk of estrogens excreted by mothers with anencephalic fetuses is derived from maternal DS. A comparison with production and conversion rates in non-pregnant women indicates that the placenta must be involved in this biotransformation (77). Thus, an impairment of the blood circulation in the intervillous space is unlikely. The normal urinary excretion of pregnanediol by pregnant women with an anencephalic fetus (2, 3, 5, 6, 35) further contradicts the hypothesis of ten Berge.

Although it cannot be ruled out at present that a combination of factors might be responsible, the decrease of fetal adrenal precursors alone seems sufficient to cause the known drop in the maternal urinary estrogens.

Measurements of conjugated D (12) and total D (23), without internal control, in normal maternal antecubital blood have lead to controversial results. Our measurements of DS utilizing 4-^{14}C-DS as internal standard revealed, in all cases, values within the range observed in normal pregnant women at delivery, with one low normal. This is in close agreement with the results of Colás et al. (23). Whether the failure to detect conjugated D in one case and the very low "probably not significant" value in another reported by Nichols et al. (12) are due to methodology or indicate exceptional cases cannot be decided. The normal maternal values of DS in our series are in agreement with the normal production rates of DS found in four pregnant women with anencephalic fetuses (7).

In regard to 16α-OH-D and its sulfate in maternal blood it should be emphasized that neither has been sufficiently identified. The values reported for normal pregnant women are very low (22) and in several instances (23) total 16α-OH-D was so low that it is questionable whether the values were different from zero. It seems that there is no difference between mothers with normal and anencephalic fetuses (4). In all instances our method was not sensitive enough to detect any maternal 16α-OH-D or 16α-OH-DS.

More work with refined methods is needed, but Browne's prophecy from 1920 has now been at least partly fulfilled: "Enough has, however, been said to show that a Gordion Knot is the problem of the interrelationship of the ductless glands, and that in the hitherto despised anencephalic monster there is available material by means of which much needed light may be shed upon many disputed questions in physiology, and pathology, and perhaps some of them even solved" (55).

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IDENTIFICATION AND MEASUREMENT OF SULPHATE-CONJUGATED NEUTRAL STEROIDS IN THE INTESTINAL CONTENTS OF EARLY AND MID-TERM FOETUSES

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SUMMARY

Sulphate-conjugated neutral steroids in the intestinal contents of early and mid-term human foetuses (14–20 weeks of gestation) were investigated by gas–liquid chromatography and gas chromatography–mass spectrometry. Twenty-four neutral steroids were found in the monosulphate fraction and 21 in the disulphate fraction. The total concentration of these steroids varied between 9·6 and 15·3 mg/100 g meconium wet weight, about half being monosulphates and half disulphates. Steroids with a 3β-hydroxy-5-ene structure were found and also saturated steroids which carried hydroxyl groups at carbons 3, 11, 15, 16, 17, 18, 19, 20 or 21. Among them were steroids not previously detected in human foetal compartments. Thus, the foetal meconium is both qualitatively and quantitatively the richest foetal source of neutral steroid sulphates so far investigated.

The present results together with those reported in the literature show that in the course of pregnancy there is a many-fold increase in the steroid concentration of the foetal intestinal contents. This is most obvious in the case of saturated C_{19} steroids and polar pregnane derivatives. Reduction of the 20-ones to 20β-ols seems to be more extensive towards the end of pregnancy. 5,16-Pregnadienes and 3β-hydroxy-5β-pregnanes are present in higher concentrations in early pregnancy.

INTRODUCTION

The meconium of a newborn is known to be a rich source of steroid hormones. Unconjugated oestriol (Kinsella, Francis, Thayer & Doisy, 1956) and its glucuronide (Menini & Diczfalusy, 1960) and sulphate (Menini & Diczfalusy, 1961) conjugates were the first to be detected. The first neutral steroids identified in this source were dehydroepiandrosterone (Francis, Shen & Kinsella, 1960), 3α-hydroxy-5β-pregn-16-en-20-one (Francis, Shen & Kinsella, 1962), 5β-pregnane-3α,20α-diol (Francis & Kinsella, 1966) and 5α-pregnane-3α,20α-diol (Francis & Kinsella, 1967). Quite recently, analyses by gas chromatography and gas chromatography–mass spectrometry have clearly shown the complexity of the steroid pattern in the meconium

(Shackleton, Gustafsson & Sjövall, 1970; Gustafsson & Stenberg, 1971; Kinsella & Francis, 1971).

The steroid pattern of the intestinal contents of early and mid-term foetuses is not known. Its analysis was felt to be of interest for the exploration of changes in steroid metabolism in the course of pregnancy. Further, such an analysis was expected to give additional information on foetal steroid metabolism and particularly on the role played by the liver. Therefore, in the present study neutral steroid mono- and disulphates in the intestinal contents of human foetuses of early and mid-gestation were identified and measured.

MATERIALS AND METHODS

Solvents were of reagent grade and were distilled twice through a 1 m fractionation column before use.

Reference steroids. Unless otherwise indicated, the reference steroids were purchased from Ikapharm, Ramat-Gan, Israel. 16β-Hydroxydehydroepiandrosterone, 3β,16α-dihydroxy-5β-pregnan-20-one, 16α-hydroxypregnenolone, 3α,21-dihydroxy-5β-pregnan-20-one, tetrahydrocorticosterone and allotetrahydrocorticosterone were obtained from the M.R.C. Steroid Reference Collection, London, England. 18-Hydroxyandrosterone was kindly supplied by Dr D. K. Fukushima, New York, U.S.A., and 3β-hydroxy-5,16-pregnadien-20-one by Dr J. Sjövall, Stockholm, Sweden.

Thin-layer chromatography (t.l.c.) was carried out on precoated abrasion-resistant silica gel F_{254} layers (Merck AG, Darmstadt, Germany) in the solvent system chloroform:ethanol (9:1, v/v, two developments) or cyclohexane:ethyl acetate (1:1, v/v, five developments) (Lisboa, 1969).

Digitonin separation was performed essentially as described by Butt, Henly & Morris (1948).

Steroid derivatives. The steroids were analysed as their trimethylsilyl (TMS) ether derivatives (Luukkainen, VandenHeuvel, Haahti & Horning, 1961). In some identification problems, *O*-methyl oxime trimethylsilyl (MO–TMS) ether (Fales & Luukkainen, 1965) and *O*-isopropylidene (acetonide) trimethylsilyl (Ac–TMS) ether (Adlercreutz, Laiho & Luukkainen, 1967) derivatives were used.

Trivial and systematic names. 18-Hydroxyandrosterone, 3α,18-dihydroxy-5α-androstan-17-one; 16α-hydroxydehydroepiandrosterone, 3β,16α-dihydroxy-5-androsten-17-one; 16β-hydroxydehydroepiandrosterone, 3β,16β-dihydroxy-5-androsten-17-one; 16α-hydroxypregnenolone, 3β,16α-dihydroxy-5-pregnen-20-one; 17α-hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one; 21-hydroxypregnenolone, 3β,21-dihydroxy-5-pregnen-20-one; tetrahydrocorticosterone, 3α,11β,21-trihydroxy-5β-pregnan-20-one; allotetrahydrocorticosterone, 3α,11β,21-trihydroxy-5α-pregnan-20-one; stigmasterol, (24*S*)-24-ethylcholesta-5,22-dien-3β-ol.

Material for analyses. Human early and mid-term foetuses (14–20 weeks of gestation) were obtained at interruption of pregnancy for socio-medical reasons. The foetus was delivered by abdominal hysterotomy. The whole intestinal tract was excised and the contents were pressed out from the intestines, weighed and dropped into acetone:ethanol (1:1, v/v).

Procedure. The sample was homogenized in 50 ml acetone:ethanol (1:1, v/v) with

an Ultra-Turrax homogenizer (Janke & Kunkel KG, Staufen i. Br., Germany). The homogenate was incubated overnight at 39 °C and then filtered through a sintered glass funnel. The precipitate was resuspended in about 30 ml acetone:ethanol and incubated for a further 2 h at 39 °C. After filtration, the combined filtrates were evaporated *in vacuo* at 39 °C. The dry residue was dissolved in 20 ml 70 % aqueous methanol and left at −20 °C for 48 h. After centrifugation the supernatant was evaporated *in vacuo*. Chromatography on a 20-g Sephadex LH-20 column was performed as described earlier (Laatikainen & Vihko, 1969*a*), and fractions were separated containing, respectively, unconjugated steroids, steroid glucosiduronates, steroid monosulphates and disulphates. The last two fractions were processed further. The sulphates were solvolysed in ethyl acetate acidified with sulphuric acid. Further fractionation according to polarity was carried out on a 3-g silicic acid column (see Laatikainen & Vihko, 1969*b*). The final purification was performed on a 200-mg silicic acid column (Vihko, 1966).

For quantitative analysis, a known amount of an internal standard (10–20 μg stigmasterol) was added to the fractions before the formation of TMS derivatives. The measurements were made as described by Vihko (1966).

For *gas–liquid chromatography* (g.l.c.) 3 % QF-1 and 2·2 % SE-30 columns and flame ionization detectors were used, as previously described (Sjövall & Vihko, 1966; Vihko, 1966). The retention times are given relative to that of 5α-cholestane (relative retention time, RRT).

For *gas chromatography–mass spectrometry* (GC–MS) an LKB 9000 instrument (LKB-Produkter AB, Stockholm-Bromma, Sweden) was used with QF-1 and SE-30 columns (Sjövall & Vihko, 1966; Vihko, 1966). The energy of the bombarding electrons was 70 eV and the ionizing current 60 μA.

A compound was considered to be identified when, on both liquid phases, the retention times and mass spectra of its TMS derivative were the same as those of the authentic compound. When needed for unequivocal characterization, other derivatives (MO-TMS or Ac-TMS) were prepared and analysed. t.l.c. or digitonin separations were used when sufficient separation was not obtained on g.l.c. alone.

RESULTS

Table 1 lists the neutral steroid mono- and disulphates identified in the analyses, and gives their RRT values and those of appropriate reference compounds. The limit of detection of a steroid in the fractions investigated was about 0·1 μg, which corresponds to a concentration of 5–10 μg steroid/100 g meconium (wet weight). Altogether 24 neutral steroids were detected in the fraction of monosulphates and 21 in the fraction of disulphates. The g.l.c. analyses of the four silicic acid fractions containing the monosulphate-conjugated neutral steroids are presented in Fig. 1.

In the following, a description is given of the identification of the sulphate-conjugated neutral steroids in the intestinal contents of early and mid-term foetuses.

Compound 1. The RRT's and mass spectra of the TMS derivative of this compound were identical with those of dehydroepiandrosterone TMS ether (Vihko, 1966).

Compounds 2 and 3. The TMS derivatives of compounds 2 and 3 gave a mass spectrum of a 5-androstene-3,17-diol di-TMS ether (Vihko, 1966). According to the

RRT values (Table 1), compound 2 was identified as 5-androstene-3β,17α-diol and compound 3 as 5-androstene-3β,17β-diol. Both the monosulphate and the disulphate fraction contained these two compounds.

Compounds 4, 5 and 6 gave mass spectra showing them to be dihydroxymono-oxo C_{19} steroids. The mass spectra of the TMS ethers of compounds 4 and 5 were very

Table 1. *Relative retention times (RRT) of the trimethylsilyl derivatives of the steroids identified in the fractions of monosulphates and disulphates of the intestinal contents of human early and mid-term foetuses*

(Cholestane = 1·00. Cholestane retention time: QF-1 = 11–12 min, SE-30 = 27–29 min. For conditions of gas–liquid chromatography see Fig. 1.)

Compound no.	Identification	Monosulphates		Disulphates		Reference steroid	
		QF-1	SE-30	QF-1	SE-30	QF-1	SE-30
1	Dehydroepiandrosterone	1·36	0·47	—	—	1·35	0·47
2	5-Androstene-3β,17α-diol	0·49	0·53	0·48	0·53	0·48	0·53
3	5-Androstene-3β,17β-diol	0·57	0·61	0·57	0·61	0·57	0·61
4	16α-Hydroxydehydroepiandrosterone	1·43	0·84	1·42	0·84	1·43	0·83
5	16β-Hydroxydehydroepiandrosterone	1·66	0·93*	1·66	0·93*	1·66	0·92
6	3β,17β-Dihydroxy-5-androsten-16-one	1·80	0·93*	1·79	0·93*	1·80	0·94
7	18-Hydroxyandrosterone	—	—	1·66*	0·71	1·68	0·71
8	5-Androstene-3β,16α,17β-triol	0·96	1·20	0·96	1·21	0·97	1·20
9	5-Androstene-3β,16β,17α-triol	—	—	0·77	1·01	0·79	1·02
10	3α-Hydroxy-5β-pregnan-20-one	1·57	0·66	—	—	1·57	0·66
11	Pregnenolone	1·81	0·78	—	—	1·81	0·78
12	3β-Hydroxy-5,16-pregnadien-20-one	1·62	0·72	—	—	1·61	0·71
13	3β,15ξ-Dihydroxy-5,16-pregnadien-20-one†	2·02	1·07	—	—	— §	— §
14	5α-Pregnane-3α,20α-diol	—	—	0·86	0·92	0·86	0·93
15	5α-Pregnane-3β,20α-diol	1·14	1·14	1·13	1·14*	1·14	1·14
16	5β-Pregnane-3α,20α-diol	0·91	0·96	0·91	0·96	0·93	0·96
17	5-Pregnene-3β,20α-diol	1·06	1·12*	1·06	1·13*	1·08	1·14
18	3β,16α-Dihydroxy-5β-pregnan-20-one	1·57	0·99	—	—	1·55	0·99
19	16α-Hydroxypregnenolone	2·02	1·28	2·00	1·28	1·97	1·26
20	17α-Hydroxypregnenolone	2·00	1·12*	2·00	1·13	1·98	1·11
21	3α,21-Dihydroxy-5β-pregnan-20-one	2·53	1·51	2·52	1·48	2·51	1·49
22	21-Hydroxyprognenolone	3·22	1·89*	3·20	1·89*	3·24	1·90
23	3β,21-Dihydroxy-5,16-pregnadien-20-one†	3·00	1·89*	3·00	1·89*	— §	— §
24	5α-Pregnane-3α,20α,21-triol	1·40	1·78	1·42	1·82	1·40	1·83
25	5β-Pregnane-3α,20α,21-triol	—	—	1·42	1·82	1·41	1·78
26	5-Pregnene-3β,20α,21-triol	—	—	1·79	2·22	1·84	2·25
27	3ξ,15ξ,16ξ-Trihydroxy-5ξ-pregnan-20-one†	1·72	1·30	—	—	— §	— §
28	3ξ,16ξ,19-Trihydroxy-5ξ-pregnan-20-one†	2·20	1·59	—	—	— §	— §
29	3β,11β,21-Trihydroxy-5β-pregnan-20-one	3·20	2·12	3·20	2·10	3·19‡	2·12‡

* Mixture of compounds.
† Tentative identification.
‡ Values calculated from the RRT values of the corresponding 5α-isomer and the 5α:5β ratio obtained from the RRT values of 3β-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnan-20-one.
§ Reference steroid not available.

similar and typical of the TMS ether derivatives of 3,16-dihydroxy-5-androsten-17-ones (Shackleton, Kelly, Adhikary, Brooks, Harkness, Sykes & Mitchell, 1968*a*; Jänne & Vihko, 1969; Siegel, Adlercreutz & Luukkainen, 1969). The RRT values (Table 1) show compound 4 to be 16α-hydroxydehydroepiandrosterone and compound 5 to be 16β-hydroxydehydroepiandrosterone. The mass spectrum of compound 6 was identical with that of the TMS ether derivative of 3β,17β-dihydroxy-5-andro-

sten-16-one (Shackleton *et al.* 1968*a*; Jänne & Vihko, 1969; Siegel *et al.* 1969) and the RRT values obtained in g.l.c. confirm this identification. Each of these three steroids was present as mono- and as disulphate.

Compound 7. This steroid was identified as 18-hydroxyandrosterone by the RRT values and mass spectra of its TMS derivative (Laatikainen & Vihko, 1969*a*). It was detected only as the disulphate and it was the only C_{19} steroid found with a saturated steroid nucleus.

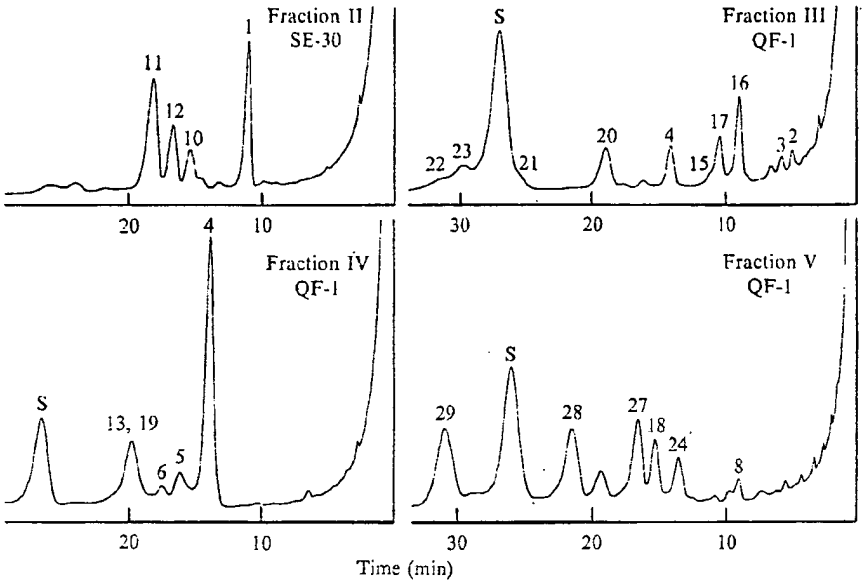

Fig. 1. Gas chromatographic analysis of the trimethylsilyl ethers of neutral steroids derived from the monosulphate fraction of foetal intestinal contents. The silicic acid fractions (II–V) are numbered in order of increasing polarity. Columns: 3 % QF-1, 2 m × 3·5 mm; 2·2 % SE-30, 2 m × 3·5 mm. For the identification of the compounds see Table 1 and the text. S = stigmasterol (internal standard).

Compounds 8 and 9 gave mass spectra characteristic of TMS derivatives of 5-androstene-3,16,17-triols (Laatikainen, 1970*a*; Jänne, 1971). The four possible isomers of 5-androstene-3β,16,17-triol are separated from each other on QF-1 and SE-30 columns (Shackleton *et al.* 1970; Jänne, 1971). According to the RRT values, compound 8 was identified as 5-androstene-3β,16α,17β-triol and compound 9 as 5-androstene-3β,16β,17α-triol. Compound 8 was detected as both monosulphate and disulphate but compound 9 as the disulphate only.

Compound 10 as its TMS ether derivative gave a mass spectrum and RRT values identical with those of the corresponding derivative of 3α-hydroxy-5β-pregnan-20-one (Eriksson, Gustafsson & Sjövall, 1968; Laatikainen & Vihko, 1969*b*).

Compound 11. This steroid was identified as pregnenolone according to the RRT values (Table 1) and typical mass spectrum of its TMS ether derivative (Sjövall & Vihko, 1968).

Compound 12. The mass spectra and RRT values of the TMS ether derivative of this compound showed it to be 3β-hydroxy-5,16-pregnadien-20-one (Gustafsson,

Shackleton & Sjövall, 1969). The possibility of artificial formation of this steroid from 16α-hydroxypregnenolone during the solvolysis of its sulphate (Bhavnani & Solomon, 1969) was excluded by treatment of 16α-hydroxypregnenolone and its disulphate with the solvolytic medium (see Materials and Methods). No formation of 3β-hydroxy-5,16-pregnadien-20-one was detected.

Compound 13. The mass spectrum of this compound (Fig. 2) has a molecular ion at m/e 474, which suggests the structure of a TMS derivative of a C_{21} steroid with two hydroxyl groups, one oxo group and two double bonds. The peak at m/e 129

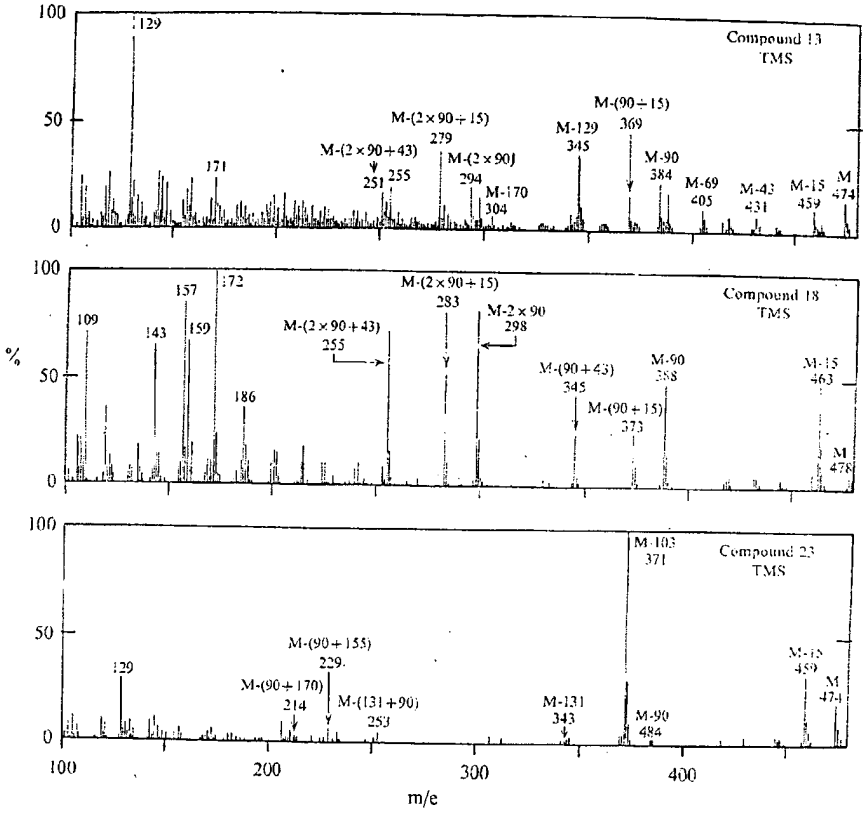

Fig. 2. Mass spectra of the trimethylsilyl (TMS) ethers of compound 13 (3β,15ξ-dihydroxy-5,16-pregnadien-20-one, tentative identification), compound 18 (3β,16α-dihydroxy-5β-pregnan-20-one) and compound 23 (3β,21-dihydroxy-5,16-pregnadien-20-one, tentative identification).

(base peak) in a TMS derivative of a C_{21} steroid is typical of a 3-*O*-TMS-5-ene structure (Eneroth, Hellström & Ryhage, 1964; Vihko, 1966). The fragment ion 431 (M-43) reflects the cleavage of the side chain at C-17 (Peterson, 1962). The ion at m/e 171 suggests cleavage of the D-ring with a $-COCH_3$ side chain, one double bond and a TMS group at C-15 or C-16 (Gustafsson & Sjövall, 1968; Jänne & Vihko, 1970*a*). The ion at m/e 405 (M-69) is possibly formed by the cleavage of the bonds between C-15–C-16 and C-17–C-13 and shows that the TMS group in the D-ring is not at C-16 but at C-15 (Jänne & Vihko, 1970*a*). A corresponding fragmentation in a steroid

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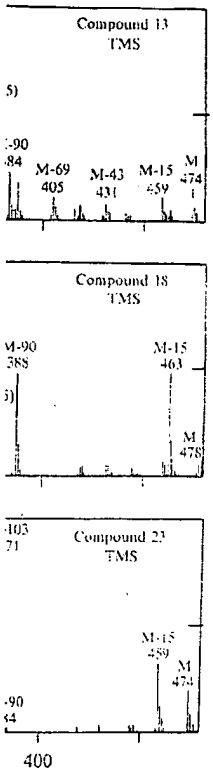

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with a saturated D-ring results in the elimination of 71 mass units (Jänne & Vihko, 1970*a*). In the present case, the size of the fragment (69 mass units) indicates a double bond between C-16 and C-17. The presence of the TMS group at C-15 and the double bond between C-16 and C-17 is further supported by the observation that the mass spectrum of the MO-TMS derivative of compound 13 also displays an ion at m/e 405 (M-98). Thus, mass spectrometric analysis very strongly suggests that compound 13 is 3β,15ξ-dihydroxy-5,16-pregnadien-20-one. This compound was present only in the monosulphate fraction.

Compounds 14, 15 and 16. These three compounds gave mass spectra with fragmentation patterns typical of pregnanediol TMS ether derivatives (Adlercreutz, Luukkainen & Taylor, 1966; Laatikainen, Peltokallio & Vihko, 1968). Of the eight possible 5α/β-pregnane-3,20-diols, 5α-pregnane-3α,20α-diol and 5β-pregnane-3β,20α-diol are not separated on the liquid phases used (Laatikainen *et al.* 1968). Compound 14 gave RRT values identical with those of these two compounds (Table 1). Compound 14 was not precipitated by digitonin, which indicates the absence of a 3β-hydroxyl group. Thus, compound 14 was identified as 5α-pregnane-3α,20α-diol. From the RRT values (Table 1) compound 15 was identified as 5α-pregnane-3β,20α-diol and compound 16 as 5β-pregnane-3α,20α-diol. 5α-Pregnane-3α,20α-diol was found in the disulphate fraction only, whereas the other two pregnanediols were present as mono- and disulphates.

Compound 17 was identified as 5-pregnene-3β,20α-diol by g.l.c. and GC–MS analyses (Vihko, 1966), and both the mono- and disulphated conjugates were present.

Compound 18. The g.l.c. data and mass spectra of this steroid as a TMS ether derivative were the same as those of 3β,16α-dihydroxy-5β-pregnan-20-one (Fig. 2). Another isomer with very similar RRT values, 3α,16α-dihydroxy-5α-pregnan-20-one, could be excluded because of the differences between the mass spectra of these two isomers (see Gustafsson, Gustafsson & Sjövall, 1968), which were confirmed in this study. 3β,16α-Dihydroxy-5β-pregnan-20-one was present only in the monosulphate fraction.

Compound 19 was identified as 16α-hydroxypregnenolone by g.l.c. and GC–MS analyses (Gustafsson *et al.* 1968).

Compound 20 as the TMS ether had g.l.c. and GC–MS properties identical with those of the TMS ether of 17α-hydroxypregnenolone (Huhtaniemi, Luukkainen & Vihko, 1970*b*).

Compound 21. The RRT values and mass spectra of the TMS and MO-TMS derivatives of compound 21 were very similar to those of 3α,21-dihydroxy-5α-pregnan-20-one or its 5β-epimer, and of 3β,21-dihydroxy-5β-pregnan-20-one (Jänne & Vihko, 1970*b*). The absence of a 3β-hydroxyl group was demonstrated by a negative digitonin test. Compound 21 and 3α,21-dihydroxy-5β-pregnan-20-one had identical mobilities on g.l.c., both as TMS (see Table 1) and MO-TMS (1·11 on QF-1 and 1·31 on SE-30 column) derivatives (Jänne & Vihko, 1970*b*). Furthermore, the two 5-epimers are separated in the t.l.c. system cyclohexane:ethyl acetate (1:1, v/v) (Laatikainen, 1970*b*). Compound 21 had a mobility identical with that of the 5β isomer. On the basis of these data compound 21 was identified as 3α,21-dihydroxy-5β-pregnan-20-one.

Compound 22. The TMS ether of this compound had the same mobilities on g.l.c.

and a mass spectrum identical with that of the corresponding derivative of 21-hydroxypregnenolone (Jänne & Vihko, 1970*b*).

Compound 23. The mass spectrum of the TMS ether derivative of this steroid (Fig. 2) had a molecular ion at m/e 474, a base peak at m/e 371 (M-103), and peaks at 343 (M-131) and 263 (M-(90+131)). The molecular weight of 474 suggests the structure of a dihydroxy mono-oxo C_{21} steroid with two double bonds. The very intensive ion at m/e 371 (M-103) is typical of a primary trimethylsiloxy group (Sharkey, Friedel & Langer, 1957) and a fragment of 131 mass units ((M-131) and M-(90+131)) indicates cleavage of the side chain with a primary trimethylsiloxy group at carbon 21 and an oxo group at C-20 (Jänne & Vihko, 1970*b*). The presence of the primary hydroxyl group at C-21 is further supported by the absence of a fragment at (M-43), which would be found in a steroid with a $-COCH_3$ side chain (Peterson, 1962). The presence of an ion at m/e 129 in the mass spectrum of a TMS derivative of a C_{21} steroid very strongly suggests a 3-hydroxy-5-ene structure for the parent compound (see Eneroth *et al.* 1964; Vihko, 1966). The compound was precipitated with digitonin. The site of the additional double bond cannot be settled with certainty. The formation of the ion at m/e 229 (possibly M-(90+155)) might include the cleavage of the side chain together with carbons 16 and 17. Further, the ion at m/e 214 M-(90+170) would be due to the rings A, B and C. These findings suggest that the additional double bond is between carbons 16 and 17. On the basis of these findings compound 23 is tentatively assigned the structure of 3β,21-dihydroxy-5,16-pregnadien-20-one.

Compounds 24 and 25. A peak with RRT values of 1·42 and 1·82 on QF-1 and SE-30 columns, respectively, gave a mass spectrum typical of a pregnane-3,20,21-triol TMS ether (Sjövall & Sjövall, 1968; Jänne & Vihko, 1970*c*). The RRT values of the TMS and Ac-TMS ether derivatives of all eight possible isomers with this structure have been published recently (Jänne & Vihko, 1970*c*). The TMS ethers of the compound(s) detected and those of 5α-pregnane-3α,20α,21-triol and 5β-pregnane-3α,20α,-21-triol have the same RRT values (Table 1). The material derived from the disulphate fraction was resolved into two compounds in the t.l.c. system chloroform:ethanol (9:1, v/v). One of them (compound 24) was shown to be 5α-pregnane-3α,20α,21-triol by comparison with the authentic compound. The other (compound 25) was similarly identified as 5β-pregnane-3α,20α,21-triol. In the same way, the material derived from the monosulphate fraction was shown to contain only the 5α-isomer. Hence it was concluded that compound 24 is 5α-pregnane-3α,20α,21-triol and compound 25 is 5β-pregnane-3α,20α,21-triol.

Compound 26. The TMS ether of this compound showed gas–liquid chromatographic and mass spectrometric properties identical with those of the corresponding derivative of 5-pregnene-3β,20α,21-triol (Jänne & Vihko, 1970*c*).

Compound 27. The TMS ether of this compound had a mass spectrum identical with that of a 3ξ,15ξ,16ξ-trihydroxy-5ξ-pregnan-20-one found in newborn meconium by Gustafsson & Stenberg (1971). The mass spectra of the TMS ethers of compound 27 and 3β,15α,16α-trihydroxy-5α-pregnan-20-one were very similar (Eriksson & Gustafsson, 1970). On this basis compound 27 is partially characterized as 3ξ,15ξ,16ξ-trihydroxy-5ξ-pregnan-20-one. This steroid was found only in the form of its monosulphate.

Compound 28 has the same mass spectrum as a compound found in newborn meconium and partially characterized as 3ξ,16ξ,19-trihydroxy-5α-pregnan-20-one (Gustafsson & Stenberg, 1971). Compound 28 is therefore partially characterized as 3ξ,16ξ,19-trihydroxy-5ξ-pregnan-20-one.

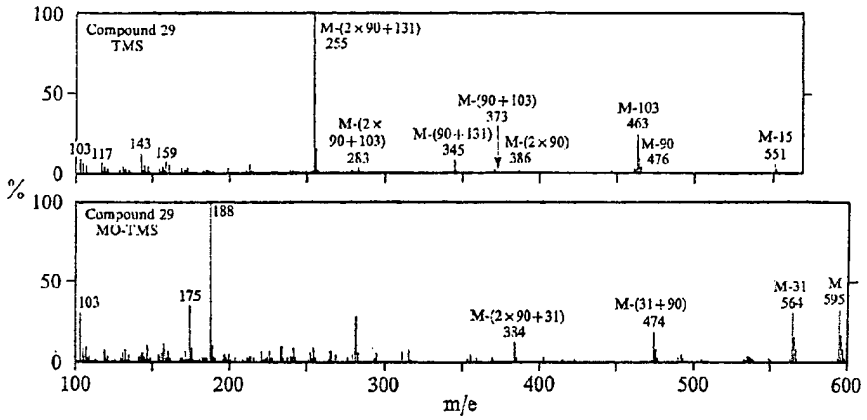

Fig. 3. Mass spectra of the trimethylsilyl (TMS) and methyl oxime trimethylsilyl (MO-TMS) ethers of compound 29 (3β,11β,21-trihydroxy-5β-pregnan-20-one).

Table 2. *Relative retention times (RRT) of the trimethylsilyl ether derivatives of the isomeric reference 3,11β,21-trihydroxypregnan-20-ones and of compound 29*

Configuration	QF-1	SE-30
5α,3α	3·22	2·27
5α,3β	4·90	3·19
5β,3α	3·26	2·27
5β,3β	3·19*	2·12*
Compound 29	3·20	2·10–2·12

* Values calculated from the RRT values of the corresponding 5α-isomer and the 5α:5β ratio obtained from the RRT values of 3β-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnan-20-one.
Conditions in gas-liquid chromatography as in Fig. 1.

Compound 29. The mass spectra of the TMS and MO-TMS derivatives (Fig. 3) of this compound were very similar to those of the corresponding derivatives of 3,11-21-trihydroxypregnan-20-ones. The mass spectrum of the trimethylsilyl ether of compound 29 has a molecular ion at m/e 566 and a base peak at m/e 255. The peaks at m/e 463 (M-103), 373 (M-(90+103)), 345 (M-(90+131)), 283 (M-(2×90+103)) and 255 (M-(2×90+131)) suggest a primary trimethylsiloxy group at carbon 21 (Sharkey *et al.* 1957) and an oxo group at C-20 (Jänne & Vihko, 1970*b*). The MO-TMS derivative of compound 29 has a molecular ion at m/e 595, and in this derivative a 21-hydroxy-20-keto structure is suggested by the prominent peaks at m/e 175 and 188 (Gustafsson & Sjövall, 1968). The RRT values of the four isomeric 3,11β,21-trihydroxypregnan-20-ones and compound 29 are shown in Table 2. From these data it is concluded that compound 29 is 3β,11β,21-trihydroxy-5β-pregnan-20-one.

Measurements. The quantities of the neutral steroid mono- and disulphate conjugates detected in the meconium samples analysed are given in Table 3. The

Table 3. *Concentrations of neutral steroid mono- and disulphates in intestinal contents of early and mid-term foetuses. The values are expressed as μg of the free steroid in 100 g meconium (wet weight) and are not corrected for methodological losses*

	Analysis I			Analysis II			Analysis III		
	MoS	DiS	Total	MoS	DiS	Total	MoS	DiS	Total
Dehydroepiandrosterone	220	—	220	310	—	310	170	—	170
5-Androstene-3β,17α-diol	20	70	90	20	260	280	10	260	270
5-Androstene-3β,17β-diol	20	40	60	20	70	90	40	50	90
16α-Hydroxydehydroepiandrosterone	440	210	650	480	390	870	490	630	1120
16β-Hydroxydehydroepiandrosterone	80	310	390	40	880	920	140	560	700
3β,17β-Dihydroxy-5-androston-16-one	180	430	610	70	670	740	140	310	450
18-Hydroxyandrosterone	—	50	50	—	120	120	—	70	70
5-Androstene-3β,16α,17β-triol	50	20	70	30	30	60	50	120	170
5-Androstene-3β,16β,17α-triol	—	30	30	—	30	30	—	120	120
3α-Hydroxy-5β-pregnan-20-one	180	—	180	390	—	390	140	—	140
Prognenolone	320	—	320	450	—	450	310	—	310
3β-Hydroxy-5,16-pregnadien-20-one	790	—	790	1330	—	1330	1290	—	1290
3β,15ξ-Dihydroxy-5,16-pregnadien-20-one†	230	—	230	300	—	300	430	—	430
5α-Pregnane-3α,20α-diol	—	130	130	—	510	510	—	450	450
5α-Pregnane-3β,20α-diol	90	150	240	160	140	300	30	200	230
5β-Pregnane-3α,20α-diol	500	590	1090	400	760	1160	210	670	880
5-Pregnene-3β,20α-diol	390	160	550	160	130	290	220	240	460
3β,16α-Dihydroxy-5β-pregnan-20-one	80	—	80	80	—	80	210	—	210
16α-Hydroxypregnenolone	240	190	430	340	430	770	420	780	1200
17α-Hydroxypregnenolone	80	20	100	190	50	240	50	30	80
3α,21-Dihydroxy-5β-pregnan-20-one	80	160	240	90	320	410	80	360	440
21-Hydroxypregnenolone	390	400	790	190	170	360	370	690	1090
3β,21-Dihydroxy-5,16-pregnadien-20-one†	320	490	810	590	820	1410	720	1120	1840
5α-Pregnane-3α,20α,21-triol	200	420*	620	320	710	1030	160	1000	1160
5β-Pregnane-3α,20α,21-triol	—			—			—		
5-Pregnene-3β,20α,21-triol	—	230	230	—	400	400	—	700	700
3ξ,15ξ,16ξ-Trihydroxy-5ξ-pregnan-20-one†	100	—	100	310	—	310	320	—	320
3ξ,16ξ,19-Trihydroxy-5ξ-pregnan-20-one†	130	—	130	610	—	610	340	—	340
3β,11β,21-Trihydroxy-5β-pregnan-20-one	310	10	320	790	10	800	540	10	550
Total	5440	4110		7670	6900		6880	8370	
Total sulphate conjugates	9550			14570			15250		

Analysis I = meconium from one foetus, 2·7 g (age 20 weeks, crown-rump length 20·0 cm). Analysis II = meconium from 5 foetuses, 1·9 g (age 14–17 weeks). Analysis III = meconium from one foetus, 2·0 g (age 13 weeks, crown-rump length 16·0 cm). MoS = monosulphates. DiS = disulphates.

* Thin-layer chromatographic separation of these two steroids was not used in the quantitative analyses of the disulphate fraction.

† Tentative identification.

tes in intestinal contents
µg of the free steroid in
logical losses

	Analysis III		
otal	MoS	DiS	Total
310	170	—	170
280	10	260	270
90	40	50	90
370	490	630	1120
920	140	560	700
740	140	310	450
120	—	70	70
60	50	120	170
30	—	120	120
990	140	—	140
150	310	—	310
130	1290	—	1290
300	430	—	430
510	—	450	450
200	30	200	230
260	210	670	880
290	220	240	460
80	210	—	210
770	420	780	1200
240	50	30	80
410	80	360	440
360	370	690	1090
310	720	1120	1840
930	160 / —	1000	1160
700	—	700	700
310	320	—	320
310	340	—	340
300	540	10	550
	6880	8370	
	15250		

) length 20·0 cm). Analysis II
meconium from one foetus,
DiS = disulphates.
ot used in the quantitative

accuracy of the method used has been tested earlier with some foetal tissues by determining the recoveries of synthetic steroid mono- and disulphates added to the tissue homogenates analysed (Huhtaniemi *et al.* 1970*b*). The mean recoveries were about 70–80 %. The figures in Table 3 have not been corrected for analytical losses.

The total concentration of sulphate conjugates was 9·6–15·3 mg/100 g of intestinal contents (wet weight) in the pools analysed. Of this, about half was in the fraction of monosulphates and half in the fraction of disulphates. The main compounds were two pregnadienes, 3β,21-dihydroxy-5,16-pregnadien-20-one (tentatively identified, 810–1840 μg/100 g) and 3β-hydroxy-5,16-pregnadien-20-one (790–1330 μg/100 g), followed by 5β-pregnane-3α,20α-diol (880–1160 μg/100 g), 16α-hydroxydehydroepiandrosterone (650–1120 μg/100 g), 16α-hydroxypregnenolone (430–1200 μg/100 g) and 21-hydroxypregnenolone (360–1060 μg/100 g).

DISCUSSION

Foetal meconium is the richest foetal source of neutral steroid sulphates so far investigated. These steroids have several routes of entry into the intestinal tract. It is possible that not all the compounds are fully absorbed from the swallowed amniotic fluid, which contains a number of sulphate-conjugated neutral steroids (see Klopper, 1970). Many of the steroids found in this study were also detected in other foetal compartments (Huhtaniemi, Ikonen & Vihko, 1970*a*; Huhtaniemi *et al.* 1970*b*; Huhtaniemi & Vihko, 1970*a*, *b*, 1972) and were possibly excreted into the intestine through the biliary tract. Yet another route would be secreta of the foetal intestinal tract itself as well as desquamated epithelial cells. In incubations (Wu, Archer, Flickinger & Touchstone, 1970) and perfusion studies (Bird, Wiqvist, Diczfalusy & Solomon, 1966) it has been shown that the intestinal epithelium is able to metabolize steroid hormones *in vitro*. The extent of the resorption of the steroids from the intestinal tract cannot be evaluated at the present moment, but it is likely that resorption takes place and might be selective, depending on the structure of the compounds.

Starting from pregnenolone and progesterone, all the enzymic activities needed for the formation of the steroids detected in this study have been found in foetal tissues. Thus, all the steroids in meconium may be products of foetal steroid metabolism. However, the possibility of placental and maternal contributions cannot be excluded.

Of the ketonic C_{19} steroids found in this study, dehydroepiandrosterone, 16α-hydroxydehydroepiandrosterone and 3β,17β-dihydroxy-5-androsten-16-one are components of the human early and mid-term plasma (Huhtaniemi & Vihko, 1970*a*). An interesting feature is the high concentration of 16β-hydroxydehydroepiandrosterone, a steroid found only in traces in other foetal tissues (Huhtaniemi & Vihko, 1972). The concentration of 5-androstene-3β,17α-diol in the meconium is higher than that of the corresponding 17β-isomer as in most other foetal compartments, such as early and mid-pregnancy plasma (Huhtaniemi & Vihko, 1970*a*), amniotic fluid (Huhtaniemi & Vihko, 1970*b*; Luukkainen, Siegel & Vihko, 1970; Jänne & Vihko, 1970*d*), term umbilical cord plasma (Shackleton, Livingstone & Mitchell, 1968*b*) and foetal kidney tissue (Huhtaniemi & Vihko, 1972). An exception is the foetal liver, where only the 17β-form was found (Huhtaniemi *et al.* 1970*b*).

One 18-hydroxylated C_{19} steroid, 18-hydroxyandrosterone, was identified in this study and it was the only saturated C_{19} steroid found. This steroid has previously been identified in human bile (Laatikainen & Vihko, 1969*a*) and may thus be of maternal origin. Foetal synthesis of this steroid, however, would seem to be possible. The human foetal adrenals are capable of converting corticosterone to aldosterone (Pasqualini, Wiqvist & Diczfalusy, 1966), a process which requires 18-hydroxylating activity. The microsomes of the human foetal liver convert testosterone to an 18-hydroxylated metabolite (Lisboa & Gustafsson, 1969). Recently, 18-hydroxyandrosterone was identified in amniotic fluid from anencephalic pregnancies (Luukkainen, Michie & Vihko, 1971), which further suggests the foetal liver as the possible 18-hydroxylating compartment.

Many of the C_{21} steroids have a 21-hydroxyl group. Of these steroids 21-hydroxypregnenolone, 5α-pregnane-3α,20α,21-triol and 5-pregnene-3β,20α,21-triol have earlier been found in amniotic fluid at term (Jänne & Vihko, 1970*d*), and 21-hydroxypregnenolone, 3α,21-dihydroxy-5β-pregnan-20-one, 5-pregnene-3β,20α,21-triol and 5α- and 5β-pregnane-3α,20α,21-triol in human pregnancy urine (Jänne & Vihko, 1970*e*). A very high 21-hydroxylating activity has been shown in human foetal adrenals (Villee, Engel, Loring & Villee, 1961; Milner & Mills, 1970) but it is also known that 21-sulphates of C_{21} steroids are able to cross the placenta without hydrolysis (Pasqualini, Cédard, Nguyen & Alsatt, 1967). Thus, the 21-hydroxylated steroids in the meconium may be of both foetal and maternal origin.

An interesting observation is the large amounts of 5,16-pregnadienes identified in foetal meconium. More than a decade ago Francis *et al.* (1962) found a 16-ene steroid, 3α-hydroxy-5β-pregn-16-en-20-one, in newborn meconium and suggested that it was an intermediate which might undergo a side chain cleavage at carbon 17. This double bond might be introduced in the foetal liver, because conversion of pregnenolone to 3β-hydroxy-5,16-pregnadien-20-one has been observed in this laboratory in incubations with human foetal liver tissue (unpublished result). On the other hand, conversion of 16α-hydroxypregnenolone to 4,16-pregnadiene-3,20-dione has been suggested to be a placental reaction (Reynolds, Wiqvist & Diczfalusy, 1969).

All the four possible 3-hydroxypregnanes with a 17β side chain were represented among the C_{21} steroids identified. It is known that various foetal tissues reduce the 4-en-3-one grouping of progesterone, favouring certain isomers. Thus, the foetal liver converts progesterone mainly into compounds with a 3α-hydroxy-5β structure, whereas lung and kidney form 3β-hydroxy-5α-pregnanes exclusively (see Diczfalusy, 1969). The formation of 3α-hydroxy-5α-pregnanes seems to be a very minor reaction in foetal tissues (YoungLai, Stern, Ling, Leung & Solomon, 1969). The enzyme activities necessary for the conversion of progesterone into a 3β-hydroxy-5β-pregnane have been found in the foetal compartment (Zander, Holzmann, von Münstermann, Runnebaum & Sieber, 1969) but no compounds with this basic structure seem to have been identified in foetal tissues. A metabolite of progesterone, 3β,16α-dihydroxy-5β-pregnan-20-one, was found in this study to be present in relatively low concentrations in foetal intestinal contents. The compound, identified as 3β,11β,21-trihydroxy-5β-pregnan-20-one sulphate and present in high concentrations, is a possible metabolite of corticosterone sulphate. In a foetal perfusion study it has been observed that the main metabolites of corticosterone sulphate are 3α,11β,21-trihydroxy-5β-pregnan-

20-one and 3β,11β,21-trihydroxy-5α-pregnan-20-one sulphates, but that the corresponding unconjugated compounds are largely reduced at position 20 and oxidized at position 11 (Pasqualini, Wiqvist & Diczfalusy, 1967; Pasqualini, Mozere, Wiqvist & Diczfalusy, 1969). This study indicates that in the metabolism of corticosterone or its sulphate, reduction to a 3β-hydroxy-5β-pregnane is of importance *in vivo* during early foetal life.

Several differences are evident between the present results on meconium of early and mid-term foetuses and those obtained by Kinsella & Francis (1971) and Gustafsson & Stenberg (1971) on meconium of the newborn. From comparisons with the concentrations of some steroids found by the latter authors, it seems that there is a tenfold rise in the concentration of neutral steroid sulphate conjugates in the foetal intestinal contents between early and mid-pregnancy and the end of gestation. Comparison of the steroid composition of the meconium of the newborn and of foetuses in early and mid-gestation shows that pregnanediols, pregnane-3,20,21-triols, 3β-hydroxy-5,16-pregnadien-20-one, 16α-hydroxypregnenolone and 16α-hydroxydehydroepiandrosterone are major components in both stages of gestation. The greatest increases seem to take place in the concentrations of androstenediols and pregnanediols during pregnancy. At both stages of development, the same ratio between total monosulphates and total disulphates (about 1:1) is seen.

In the newborn meconium there are many C_{19} steroids with a saturated steroid nucleus but only 18-hydroxyandrosterone was found in the intestinal contents in early and mid-pregnancy. No steroids with a 6β- or 7-hydroxy group were detected nor C_{19} steroids with an 11β-hydroxy of 11-oxo group. Only traces of two pregnane-3,16,20-triols and one pregnanetetrol were detected, but because of the small concentrations further attempts to identify these compounds were unsuccessful. No compounds with a 20β-hydroxyl group could be detected either. Nor could 17α-hydroxylated steroids of the pregnane series be identified.

Of the steroids detected in this study, 18-hydroxyandrosterone, 3β,16α-dihydroxy-5β-pregnan-20-one, 3α,21-dihydroxy-5β-pregnan-20-one, 3β,11β,21-trihydroxy-5β-pregnan-20-one, 3β,15ξ-dihydroxy-5,16-pregnadien-20-one, 3β,21-dihydroxy-5,16-pregnadien-20-one and 5α-pregnane-3α,20α,21-triol were not identified in the newborn meconium, and of these steroids 3β,11β,21-trihydroxy-5β-pregnan-20-one, 3β,15ξ-dihydroxy-5,16-pregnadien-20-one (tentatively identified) and 3β,21-dihydroxy-5,16-pregnadien-20-one (tentatively identified) seem to be detected for the first time in biological material.

Summarizing the observations in the steroid patterns in the meconium of early to mid-pregnancy and at term, some conclusions can be drawn. There is a many-fold increase in the steroid concentrations, especially in the saturated C_{19} steroids and polar pregnane derivatives. 20β-Reduction of the 20-oxo group seems to take place more actively towards the end of pregnancy, whereas 5,16-pregnadienes and 3β-hydroxy-5β-pregnanes seem to be more abundant in the early months.

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GENERAL REVIEW

16-UNSATURATED C_{19} STEROIDS A REVIEW OF THEIR CHEMISTRY, BIOCHEMISTRY AND POSSIBLE PHYSIOLOGICAL ROLE

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1. INTRODUCTION

SINCE THE isolation of three 16-unsaturated C_{19} steroids from pigs' testes in 1944 [1] and from human urine in 1950 [2], this group of compounds was not intensively studied until some ten years ago. With their curious musk-like odour (see p. 93) they were considered until recently biochemical oddities that neither fitted into the normal pattern of steroid biosynthesis nor had any physiological activity. This review* surveys current knowledge concerning the chemical synthesis, occurrence, metabolism and physiological role of these compounds, which are now known to be important in the pig and possibly also in humans. Some reference is also made to a phenolic 16-unsaturated steroid, oestratetraenol† since this is related metabolically to androstadienone (see p. 72).

2. OCCURRENCE

The structures of the 16-unsaturated C_{19} steroids, that have so far been isolated from natural sources, are shown in Fig. 1.

(a) *In pigs*. The first report of the presence of 16-unsaturated steroids in pigs' testes was by Prelog and Ruzicka [1] who isolated and characterised relatively large amounts of an-α and an-β. At that time it was difficult to explain why such large quantities of these substances occurred in pigs' testes while the amount of androgenic substances was so small, but more recent work [3–6] has shown conclusively that the 16-unsaturated steroids are quantitatively more important than the androgens in the pig. Indeed, it appears that the metabolism of the C_{21} steroids, pregnenolone and progesterone is biased in favour of the formation of 16-unsaturated steroids rather than androgens [6]. Prelog and Ruzicka [1] were unable to give estimates of the amounts of an-α and an-β present in their pig testes extracts because of analytical losses but later workers [3, 4] have attempted to correct the yields obtained for analytical losses and these values are shown in Table 1.

It is clear that the proportions of the 16-unsaturated steroids in boar testes vary considerably with the age of the animal. Booth (personal communication) has shown that, in 84 day old foetal testes, andien-β is the dominant steroid followed by androstadienone > an-α > an-β > 5α-androstenone. In testes at birth

*This review of the literature was concluded in May 1971.

†Abbreviations and trivial names: an-α: 5α-androst-16-en-3α-ol; ae-α: 5β-androst-16-en-3α-ol; andien-β: 5,16-androstadien-3β-ol; an-β: 5α-androst-16-en-3β-ol; 5α- and 5β- androstenones: 5α- and 5β- androst-16-en-3-ones; androstadienone: 4,16-androstadien-3-one; oestratetraenol: 1,3,5(10),16-oestratetraen-3-ol; 20β-dihydropregnenolone: 5-pregnene- 3β,20β-diol; 16-dehydroprogesterone: 4,16-pregnadiene-3,20-dione.

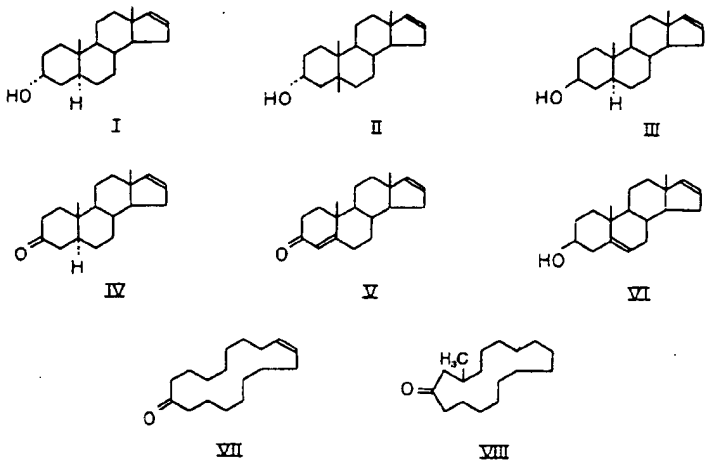

Fig. 1. Structures of some 16-unsaturated C_{19} steroids that have been isolated from natural sources. I, an-α; II, ae-α; III, an-β; IV, 5α-androstenone; V, androstadienone; VI, andien-β. The structures of civetone (VII) and muscone (VIII) are included for comparison.

Table 1. Amounts (μg/g tissue) of 16-unsaturated C_{19} steroids and testosterone present in boar tissues

Tissue	Age of boar (days)	5α-androstenone	an-α	an-β	andien-β	androstadienone	testosterone	Reference
Testis	175	0·10	0·28	1·35			0·006	[4]
	217	0·27	0·74	4·41			0·021	
	229	0·31	0·89	3·43			0·037	
	252*	0·034	0·78	2·74	0·22	0·006	0·02	W. D. Booth (personal communication)
	252†	0·05	0·34	1·62	0·10	0·006	0·031	
	4 years	0·05	0·60	3·29	0·45	0·012	0·007	
Parotid gland	175	0·17	29·9	—				[4]
	217	11·43	82·0	5·69				
	229	1·99	49·9	1·44				
Sub-maxillary gland	84	0·018	0·12	0·029	trace		trace	[3,61] and W. D. Booth (personal communication)
	252*	0·063	1·12	0·19	0·032		0·007	
	252†	0·032	0·35	0·07	0·028		0·013	
	4 years	1·08	24·3	0·116	0·098		0·028	
Fat	175	1·03						[4]
	217	7·49						
	229	1·74						

*weight 126 kg.
†weight 100 kg.

androstadienone predominates followed by andien-β > an-α > an-β > 5α-androstenone. The predominance of an-α over an-β continues in the prepubertal animal (to the age of approximately 12 weeks) but by about 18 weeks there is a reversal in the an-α:an-β ratio, an-β then becoming the predominant steroid. Table 1 shows that the ratio of an-β to an-α in animals 175–229 days is 4–6:1. These results clearly demonstrate the development with age of the biosynthetic pathway for 16-unsaturated C_{19} steroids.

In keeping with these findings, recent work [7] has shown that andien-β can be formed from pregnenolone in testes taken from pigs as young as 3 weeks and that

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Table 2. Ratios

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Submaxillary gland	2
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†weight 126
‡weight 100

the yield obtained *in vitro* is of the same order as that in adult testis tissue (see p. 66). It also seems likely that andien-β may be the first 16-unsaturated steroid formed from pregnenolone and that it is further metabolised to androstadienone and then to an-α and an-β[8]. The finding of only trace quantities of andien-β sulphate in boar spermatic vein plasma lends support to the idea[5]. The predominance of the yields of an-β over an-α in *in vitro* incubations of mature testis tissue[6, 9] (see p. 56) is in complete agreement with the results of Booth mentioned above. It is of particular interest that the reversal of the ratio of an-α to an-β occurs at puberty in the boar. Since spermatogenesis begins at this time, it is conceivable that the seminiferous tubules may be involved in this transformation. No studies have yet been performed, however, owing to the problems involved in the separation of the tubules from interstitial tissue of boar testis[10] although the seminiferous tubules in other species are capable of some steroid transformations [11].

The data collected in Tables 1 and 2 also show the extent to which 16-unsaturated steroids occur in boar testes compared with testosterone. The ratios of an-β to testosterone are as much as 100–200:1 and strongly suggest that this group of 16-unsaturated steroids may possess a special physiological significance in the boar. The relevant biochemical and physiological studies are reviewed later on pp. 56 and 96.

The amount of testicular 5α-androstenone, a compound with an intense urine-like smell (see p. 93), also increases with age[3, 4]. It is this compound which, once formed in the testes[12] passes into the spermatic venous blood[5] and thence, presumably because of its high lipid solubility, to the adipose tissue. It has long been known that bacon joints, taken from entire male pigs, emit an unpleasant smell on being cooked; Patterson[13] was the first to isolate the so-called "boar taint" from fat samples and to show that its structure was identical with that of 5α-androstenone (see Fig. 2). A quantitative analysis of fat samples from immature pigs and from male castrates, however, showed that there was little if any 5α-androstenone present, a finding in keeping with the testicular origin of this

Table 2. Ratios* of 16-unsaturated C_{19} steroids to testosterone isolated from boar testis and submaxillary glands

Tissue	Age of boar (days)	5α-androstenone / testosterone	an-α / testosterone	an-β / testosterone	andien-β / testosterone	androstadienone / testosterone	Reference
Testis	175	17	47	225			[4]
	217	13	35	200			
	229	9	24	94			
	252†	1·7	39	137	11	0·3	[3]
	252‡	1·6	11	52	3·1	0·2	
	4 years	7	85	470	65	1·7	
Submaxillary gland	252†	9	160	28	4·6		W. D. Booth (personal communication)
	252‡	2·5	27	5·7	2·2		
	4 years	33	870	4	2·5		

*ratios calculated from data in Table 1.
†weight 126 kg.
‡weight 100 kg.

compound[3,4,12]. Fat taken from boars aged 175–225 days contained up to 11·4 μg/g of 5α-androstenone but there was no age-dependence[4] (Table 1). The practical implications of the presence of this steroid for the pig will be discussed later (p. 96).

Until recently the salivary glands in animals were considered as unimportant in steroid metabolism but there is evidence now to implicate them in corticosteroid [14], androgen[15] and 16-unsaturated steroid metabolism. Patterson[16] was the first to isolate an-α from boar submaxillary gland but was unable to demonstrate its presence in the corresponding parotid or sublingual glands. Gower and Katkov [7] were able to show the formation of andien-β from pregnenolone in homogenates of boar sub-maxillary glands (see p. 63). Recently, a gas–liquid chromatographic (g.l.c.) method has been used[4] to analyse parotid glands from boars and has revealed the presence of an-α, an-β and 5α-androstenone (Table 1).

Boar saliva has also been analysed for 16-unsaturated steroids[16,17] and both an-α (approximately 1·0 μg/ml) and 5α-androstenone (approximately 0·05 μg/ml) were shown to be present. The possible implications of these findings for the physiology of the boar will be discussed in a later section (p. 96).

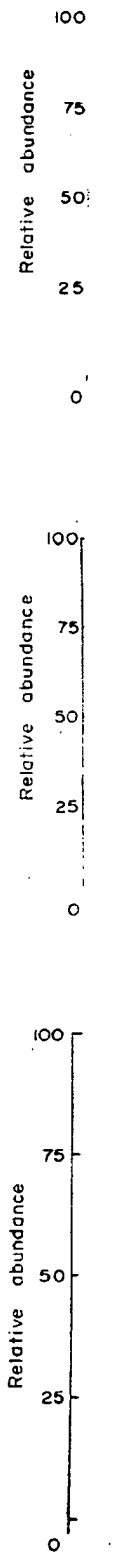
In accord with the finding of an-α, an-β and 5α-androstenone in boar testes, the same three compounds have been detected in boar spermatic vein blood[5] and characterised by column, thin-layer and gas–liquid chromatography and finally by mass spectrometry[18] (Fig. 2). The two alcohols occurred predominantly as sulphates whereas the ketone was obtained in the "free steroid" fraction. The concentration of this ketone has been estimated in peripheral plasma of boars, sows and male castrates[4,19] and a definite age- and sex-dependence was shown to exist (Table 3). There was also a possible correlation with the concentration of testosterone in the same plasma samples (Fig. 3) but so far there is no evidence that the ketone is derived from testosterone[6].

An analysis of boar urine[20,5] revealed the presence of an-β, conjugated as glucosiduronate, at a concentration of approximately 250 μg/l. A corresponding analysis of the sulphate fraction showed that no 16-unsaturated steroids were present. These results, concerning the occurrence of 16-unsaturated steroids in the pig, are summarized in Table 4.

(b) *In man.* In contrast to the pig, the predominant 16-unsaturated steroid in human urine is an-α. This was originally isolated from the hydrolysed glucosiduronate fraction of male and female urine[2]. Using a colorimetric method[21] de-

Table 3. Concentration (ng/ml) of 5α-androstenone and testosterone in the peripheral plasma of pigs (from Ref. 4)

Type	Age (days)	5α-androstenone	testosterone
Boars	175	6·0	9·6
	217	17·6	15·0
	229	22·3	16·1
Sows	175	0·8	0·4
	229	2·0	1·4
	238	2·0	1·9
Male castrates	175	2·7	1·5
	217	1·7	2·1
	236	1·3	2·4



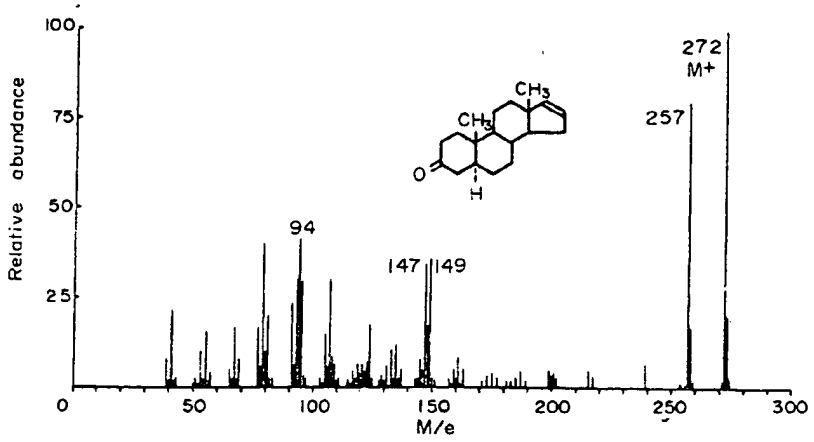
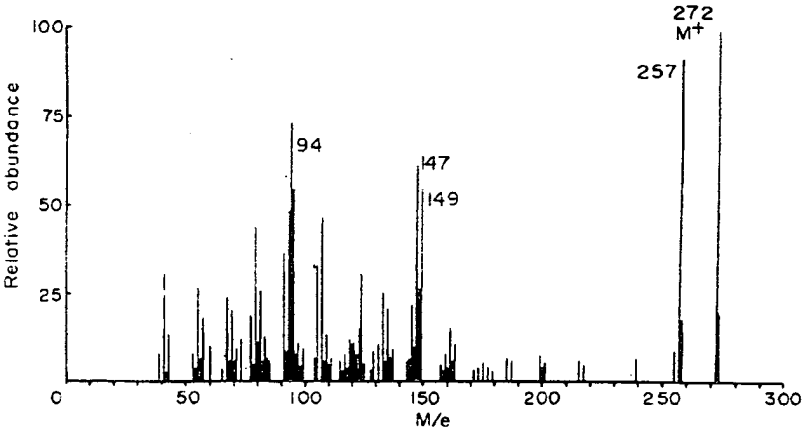


Fig. 2(A).



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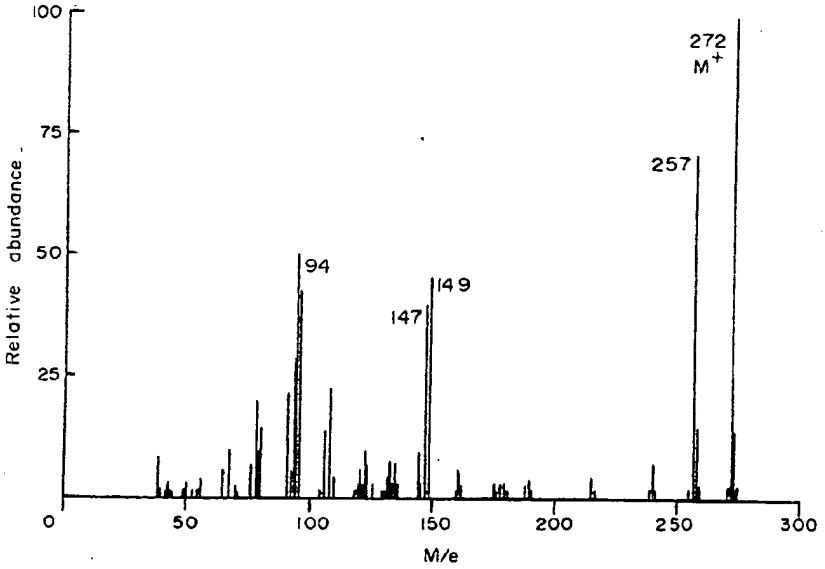

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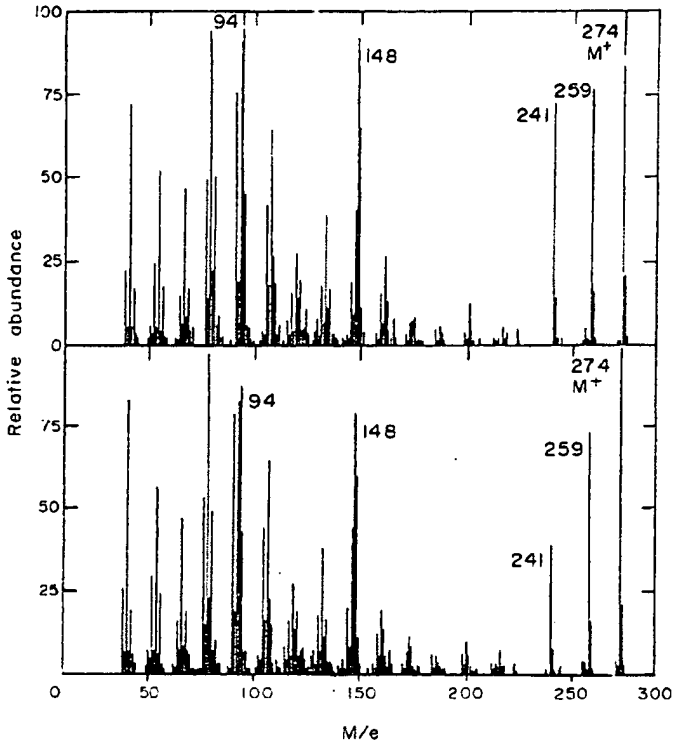

Fig. 2(D upper) and (E).

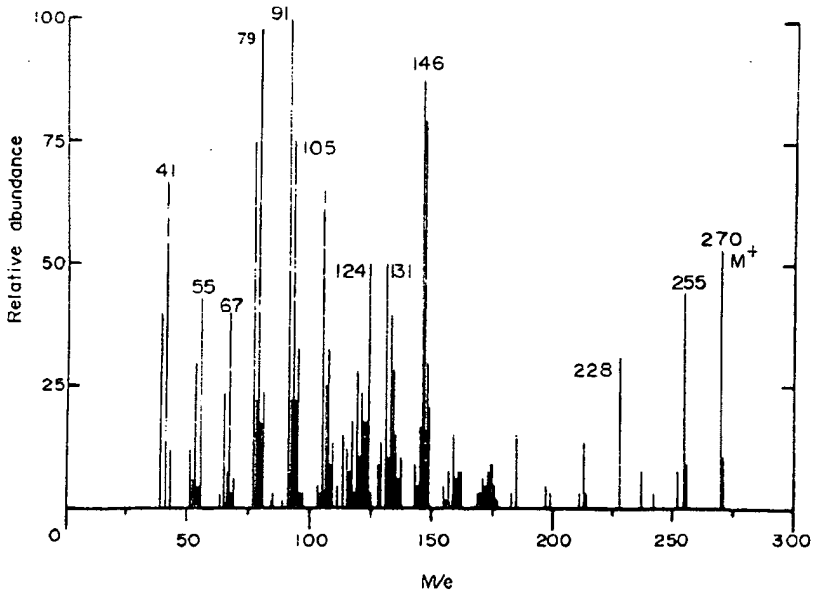

Fig. 2(F).

vised in later work (see p. 86), Brooksbank[24] analysed a large number of 24 h urine samples from healthy men and women of different ages and showed that the mean excretion was 1180 μg/day in men (age range 16–45 yr) and 429 μg/day in women (age range 16–45 yr). These results were confirmed by later investigators

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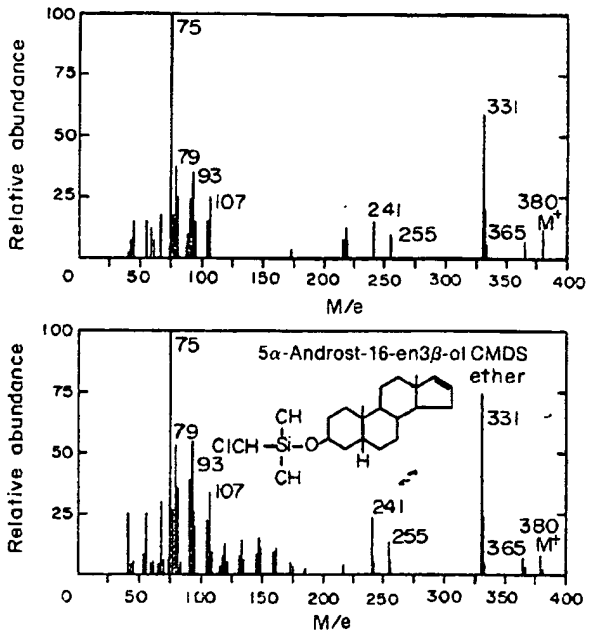

Fig. 2G (upper) and (H).

Fig. 2. Mass spectra of 16-unsaturated C_{19} steroids. A, 5α-androstenone; B, 5α-androstenone from boar fat; C, 5α-androstenone from human axillary sweat; D, an-α; E, an-β (note the different ratio of the intensities of the peaks m/e 241 : 274); F, androstadienone; G, an-β isolated from boar spermatic vein plasma and converted to the chloromethyl dimethyl silyl (CMDS) ether; H, an-β CMDS ether. (A and B are reproduced by permission of J. & A. Churchill, Ltd. from Ref. [130]; C, data from Ref. [34]; D and E by permission of The Society for Chemical Industry, Ref. [16]; F, from Gower and Patterson, unpublished [cf. Ref. 18]; G and H by permission of Journal of Endocrinology, Ltd., Ref. 18).

Table 4. Summary of 16-unsaturated C_{19} steroids present in boar tissues

Source	16-unsaturated C_{19} steroid present	Reference
Testis	an-α, an-β, 5α-androstenone, andien-β, androstadienone	[1.3.4] and personal communications from W. D. Booth and R. Claus
Salivary glands: submaxillary	an-α, an-β, 5α-androstenone, andien-β	[3.16] and W. D. Booth (personal communication)
parotid	an-α, an-β, 5α-androstenone	[4]
sublingual	none present (as judged by smell)	[16]
Saliva	an-α, 5α-androstenone	[16.17]
Back fat	5α-androstenone	[4.13]
Preputial gland	Only traces present	[65]
Spermatic vein plasma	an-α*, an-β* and andien-β (trace) as sulphates; an-α and 5α-androstenone as free steroids	[5]
Peripheral plasma	5α-androstenone	[4.19]
Urine	an-β (as glucuronoside)†	[5]

*predominantly as sulphate.
†approx. 250 μg/l.

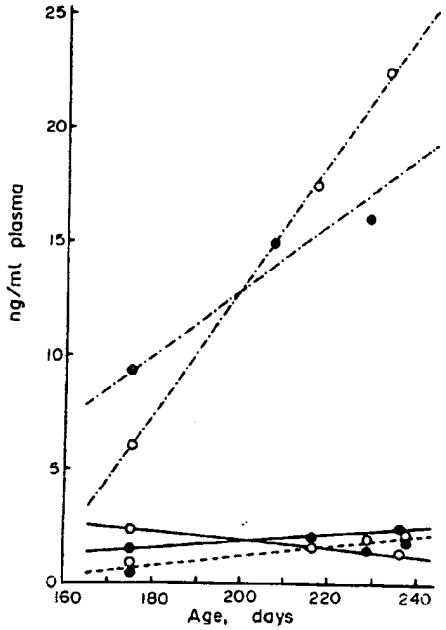

Fig. 3. Correlation with age and sex of 5α-androstenone (○) and testosterone (●) in the peripheral plasma of pigs. Males —·—·—, male castrates, ———, females ------ (data from Ref. [4] by permission).

using the same colorimetric method [22] although a g.l.c. method devised later [23] resulted in slightly lower mean values for an-α excretion (1052 and 360 μg/day for men and women respectively in the same age ranges as above). Urinary an-α excretion is very small in infants and children [22, 24] but increases at puberty to a maximum in the young adult years for both men and women and thereafter decreases to lower values in old age (Fig. 4). In many postmenopausal women an-α excretion was found to be as low as 100 μg/day or less whereas men over the age of 45 yr excreted some 500 μg/day on the average. Although the ranges for an-α excretion are very large (< 100–2630 μg/day for men and < 100–1100 μg/day for women) (Table 5), these changes with sexual development suggest a possible relationship with androgen production. An-α excretion is increased in some men after HCG administration while ACTH causes an increase in urinary an-α in both men and women [22, 24] suggesting that the source of an-α may be, in part, the testes in men and the adrenals in women.

In breast cancer patients, a positive correlation was shown to exist between urinary excretion of an-α and that of androsterone, aetiocholanolone and DHA [26]. However, there is no evidence that an-α is derived from testosterone, DHA or other C_{19}-steroids (see p. 64) nor is there any clear correlation in a group of hirsute patients [23] (Table 6).

In 1964, an investigation [27] of 69 male psychiatric patients, aged 18–45 yr, showed that they were excreting significantly less an-α than a control group of healthy men in the same age range and also less than a group of psychiatric patients in whom no signs of schizophrenia were evident. There was, however, no significant difference between the amounts of 17-oxosteroids (17-OS) excreted by the three groups of individuals. The fact that there was a polynomial, or exponential, relationship between urinary an-α and 17-OS in both the healthy subjects and

Table 5. Urinary an-α excretion (μg/24 h) in normal humans

Type	No. of subjects	Age (yr)	Mean	Range	Type	No. of subjects	Age (yr)	Mean	Range	Reference
Males	48	12–80	1008	19–2630	Females	27	15–72	430	62–1010	(21)
Males	82	12–82		< 100–2630	Females	61	12–86		< 100–1100	(24)
	61	16–45	1180 ± 78 (S.E.)†			42	16–45	429 ± 41 (S.E.)†		
Males	17	Adult	950	210–2050	Females	16	Adult	420	0–910	(22)
Males	12	20–40	1052*	17–1659*	Females	8	20–35	360*	230–420*	(23)
Pre-pubertal children	5	4–11	<20	< 10–53						(21)
Pre-pubertal children	20	4–11	< 50	< 10–90						(24)
Pre-pubertal children	14		0	0–120						(22)

Estimations were performed using the colorimetric method (21) except for those marked * where the g.l.c. method [23] was used.
†Standard Error.

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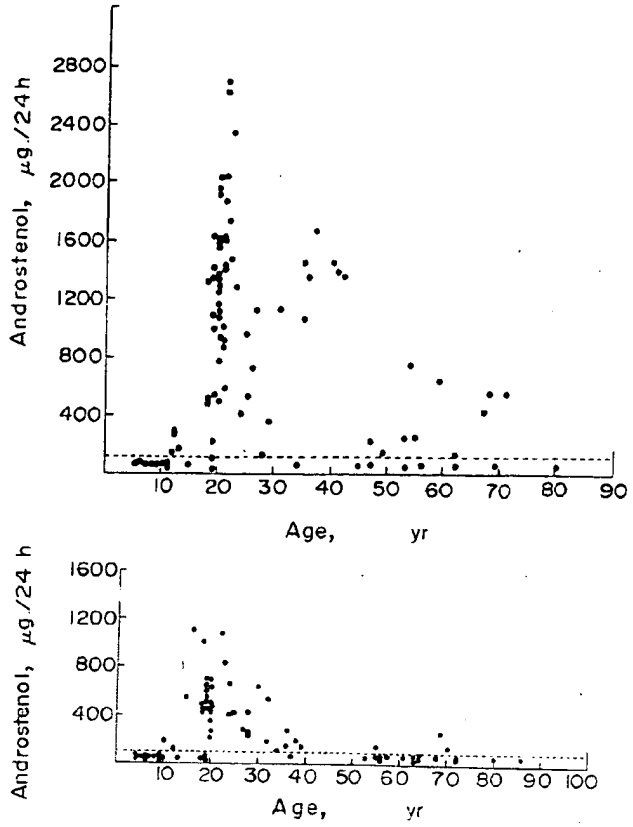

Fig. 4. Variations in urinary an-α with age in men (upper) and women (lower). Redrawn from Ref. [24] by permission of Journal of Endocrinology Ltd. The limit of reliability of the method of estimation (100 μg/24 h) is indicated thus, -------.

Table 6. Urinary an-α and 17-oxosteroids (mg/24 h) in females with simple hirsutism

Patient	Age (yr)	An-α	Androsterone	Aetichol-anolone	DHA
M.C.	32	0·7	4·8	4·8	0·95
J.W.	24	0·9	5·4	6·3	3·1
P.H.	17	0·55	4·0	3·7	6·0
I.B.	27	1·44	5·0	2·3	0·4
D.C.	40	0·43	3·3	3·3	1·75
C.M.	23	0·26	0·3	0·85	0·1
S.S.	19	0·35	2·5	1·1	0·65
P.O.	20	0·42	3·5	2·6	0·33
C..	19	0·47	1·6	1·6	0·4
S.C.	21	0·47	—	—	—
P.W.	19	0·39	—	—	—
Mean normal values (with ranges)		0·36* (0·23–0·42)	1·4† (0·9–2·6)	1·8† (0·6–2·2)	0·56† (0·23–0·42)

*From Ref. [23].
†From Ref. [31] (by permission of the publishers' Periodica, Copenhagen).

the patients was taken to suggest a possible indication of a 'safety-valve' mechanism; that is to say, the production of an-α or other 16-unsaturated steroids may be a side-reaction which becomes of greater importance as androgen production rates increase. At low androgen production rates this mechanism would not be expected to operate. As discussed by Brooksbank and Pryse-Phillips[27] the existence of such a mechanism would imply that an-α excretion would be a more sensitive index of effective androgen secretion than that of 17-OS. In a more recent study[28], however, the urinary an-α levels of a smaller group of schizophrenics (10 individuals) were substantially higher than in the earlier study and there was now no difference between the urinary an-α levels of patients and controls. The reason for the failure to repeat the earlier observations[27] may be the existence of a sub-group of schizophrenics in whom signs of androgen deficiency are present. It is conceivable that, in the earlier study, the patient group contained more individuals of this type.

In addition to an-α, the glucuronoside fraction of human urine contains two more 16-unsaturated C_{19} steroids, ae-α and andien-β, at concentrations of approximately 6 and 25 μg/l respectively[29]. More recent analyses[23] have resulted in mean values (μg/g creatinine) for ae-α and andien-β of 5·88 and 43·4 respectively for men and 4·77 and 20·7 respectively for women. In these studies no an-β has been detected in human urine but recently the presence of a small amount has been demonstrated following the intravenous administration of labelled androstadienone[30]. In a woman with a virilizing adrenocortical carcinoma[31] approximately 30% of the urinary an-α was excreted conjugated as sulphate (see p. 65).

Human plasma has received scant attention as far as 16-unsaturated steroids are concerned. Brooksbank and his colleagues[32] have provided evidence for the presence of small quantities of androstadienone in male peripheral plasma but only approximate values were given by these workers since no correction was made for analytical losses entailed by the method. The amount of androstadienone in the 'free steroid' (ether-extractable) fraction was approximately 0·5–1·0 μg/l, while twice this amount was present in the 'sulphate-conjugated' fraction. From experiments performed subsequently both in Brooksbank's and in the author's laboratories, it seemed likely that some androstadienone existing as 'free steroid' in plasma was not extracted with ether at an alkaline pH and this may thus have contributed to that recovered after solvolysis of sulphates. More recent analyses of pools of plasma from normal men and women, using [^{3}H]-androstadienone to estimate losses, have given values in the free fraction of 0·984 and 0·366 μg/l respectively[33]. The only other reference to 16-unsaturated steroids in human plasma is by Gower and Stern[31] who reported tentative evidence for the presence of an-α in the peripheral plasma of a woman with a virilizing adrenocortical carcinoma; after adrenalectomy, an-α was no longer detectable.

In view of the intense smell of some of the 16-unsaturated C_{19} steroids, especially 5α-androstenone (see p. 93), Gower and Llewellyn[34] felt it worthwhile to analyse axillary sweat obtained from human male subjects to see if these compounds could contribute to underarm smell. Collections of axillary male sweat were made for periods of up to 18h on pads of fat-free cotton-wool strapped in the arm-pits. The pads were changed at intervals of six hours and the combined pads extracted in a Soxhlet apparatus with double-distilled peroxide-free ether. After removal of fatty acids, by washing with 0·1 M NaOH, the ether extract was

dried and evaporated to dryness. The residue was then subjected to sublimation on a micro-scale at 90°C under a vacuum of 0·15 mmHg. The lipid that had condensed on the cold-finger (maintained at −67°C throughout) was subsequently recovered with ether and the solution analysed by g.l.c. and finally by combined gas chromatography-mass spectrometry. 5α-Androstenone was thus characterised in one male axillary sweat sample (Fig. 2), but attempts to detect this same compound in the sweat of other male subjects have failed (B. W. L. Brooksbank, personal communication). It is conceivable that there may be a temporal or individual variation in the excretion by this route of this powerfully-smelling compound. After intravenous administration of [4−^{14}C] androstadienone to men or women, the axillary excretion of ^{14}C was slight and no greater than after administration of labelled 4-androstenedione, progesterone or pregnenolone by the same route (Ref. 35 and B. W. L. Brooksbank, personal communication).

The phenolic 16-unsaturated steroid oestratetraenol is formed from androstadienone (see p. 72) and occurs in pregnancy urine (conjugated as glucosiduronate) at a concentration of approximately 100 μg/l [36].

3. BIOSYNTHESIS OF 16-UNSATURATED STEROIDS *IN VITRO*

(a) *In animals other than pigs.* In 1960 the presence of an-α and an-β in pigs' testes was known [1] and so also was the occurrence of an-α in human urine [2]. At that time, however, nothing was known about the biosynthesis of these compounds although it was generally thought that testosterone was the parent compound. This hypothesis was supported by evidence that testosterone was converted in small yield to androstadienone in a rat testis homogenate [37] and to androstadienone together with an-β in a human liver preparation [38]. At that time an-α was believed to be a specific urinary metabolite of testosterone [39].

In the years following 1960 in the author's laboratory, experiments were designed in order to ascertain the biosynthetic origin of 16-unsaturated steroids in, initially, rats, rabbits and guinea-pigs. An-α was formed in small yield from acetate in testis slices of rabbits and guinea-pigs [40]. In later experiments the metabolism of pregnenolone [41] and DHA [42] to 16-unsaturated steroids was studied in detail in rat testis preparations. Pregnenolone was converted to small yields of androstadienone and ae-α [41] while these compounds as well as an-α were formed from DHA [42]. A kinetic study of the formation androstadienone from DHA showed that the former increased in amount up to 2 h incubation but thereafter remained constant [42]. It should be emphasized, however, that the yields of 16-unsaturated steroids obtained in most of these experiments (summarized in Table 7) were extremely small (approximately 0·1% or less) and it seems unlikely that this group of compounds is of great significance in rabbits, rats or guinea-pigs. In contrast, bullock adrenal slices produced higher yields (1–2%) of an-α from DHA acetate [43].

(b) *Biosynthesis in porcine tissues.* Since 16-unsaturated steroids had been isolated from porcine testis by Prelog and Ruzicka [1], Gower and his collaborators considered it worthwhile to investigate the biosynthesis of these compounds in boar testis preparations. Both the C_{19} steroids pregnenolone [9] and progesterone [6] were metabolised in boar testis preparations to high yields of an-β (10–15%) and also to an-α (1–2%). These yields at once suggested that the 16-unsaturated steroids might be of particular importance in the boar, especially as the yields of androgenic compounds were relatively small in the same incubations [6]

Table 7. Biosynthesis *in vitro* of 16-unsaturated steroids in animals other than pigs

Species	Tissue preparation	Cofactors	Precursor	16-unsaturated steroid formed	Yield (%)	Reference
Rabbit, guinea-pig	testis slices	nicotinamide, ATP	acetate	an-α	0·05–0·1	[41]
Rat	testis homogenate	$NADP^+$, NADPH-generating system	testosterone	an-β androstadienone	~ 0·5 ~ 0·5	[37]
Bull	adrenocortical slices	nicotinamide, ATP	DHA acetate	an-α	1–2	[43]
Rat	testis mince	NAD^+, $NADP^+$, ATP, glucose	pregnenolone	ae-α androstadienone	0·1 0·02	[41]
Rat	testis minces (kinetic study)	NAD^+, $NADP^+$, ATP, glucose	DHA	an-α*, ae-α* androstadienone	0·1	[42]
Rabbit, rat, bull	testis minces	NAD^+, $NADP^+$, ATP	testosterone epitestosterone	none		[45]
Rat	liver 100,000 g supernatant; liver microsomes	NADH or NADPH NADH or NADPH	testosterone epitestosterone	none none		[46]

*tentative identification.

(Table 8). Recent work[3, 5] (already mentioned on p. 47) has also shown that the quantities of 16-unsaturated steroids in boar testis are greater than those of other C_{19} steroids such as testosterone. A more detailed kinetic study[6, 44] of the metabolism of progesterone in boar testis minces (Fig. 5) suggested that androstadienone might be the first 16-unsaturated steroid formed, being subsequently converted to the ring A-saturated alcohols, an-α and an-β, that accumulated later in the incubation period. In the light of more recent work, it seems likely that progesterone gives rise only to traces of androstadienone whereas some ten times as much is formed from pregnenolone under the same incubation conditions[6, 8] (Table 8) and very similar results have been obtained in human testis tissue[71] (Table 13). Evidence has also been provided[8] for the formation of andien-β in high yield (15–20%) from pregnenolone in boar testis

Table 8. Percentage incorporation of ^3H and ^{14}C into 16-unsaturated steroids and other metabolites obtained after incubation of ^3H- and ^{14}C-labelled C_{21} steroids with minces of boar testis

	Incubation 1 ([4-^{14}C]pregnenolone and [7α-^{3}H]progesterone)			Incubation 2 ([7α-^{3}H]progesterone and 17α-hydroxy[4-^{14}C]progesterone)		
Metabolite	^3H(%)	^{14}C(%)	$^3H/^{14}C$ ratio	^3H(%)	^{14}C(%)	$^3H/^{14}C$ ratio
Androstadienone	0·007	0·064	0·1	0·006	0	—
An-α	1·92	1·62	1·1	1·5	0	—
An-β	11·1	10·6	1·1	10·0	0	—
Androstenedione	0·46	0·86	0·5	0·21	0·33	0·63
Testosterone	0·37	1·23	0·3	0·32	0·53	0·6
17α-Hydroxyprogesterone	0·014	0·005	0·3	0·07	0·75	0·1
Progesterone	2·17	0·011	197	1·9	0	—
Pregnenolone	0	9·0	—	0	0	—
DHA	0	0·007	—	0	0	—

Results are expressed as percentages of the radioactivity extracted from the equivalent of 1 g wet wt. of tissue, after correction for analytical losses.

Data from Ref. [6], by permission of The Biochemical Journal.

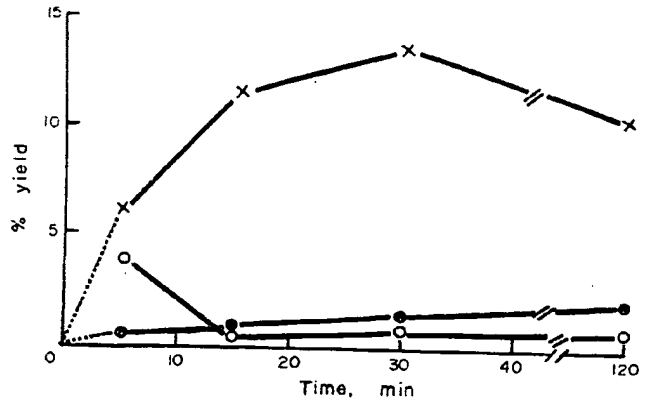

Fig. 5. Kinetic study of the formation of 16-unsaturated C_{19} steroids from [4-^{14}C] progesterone in a boar testis mince. Data from Refs. [6] and [44]. Androstadienone(○), × 100; an-α( ); an-β(×).

homogenates. Since andien-β could be converted to androstadienone if NAD^+ was present[8], it seemed that there were two possible pathways for androstadienone formation from pregnenolone:

(i) pregnenolone → andien-β → androstadienone

and

(ii) pregnenolone → progesterone → androstadienone.

Pathway (ii) however, appeared to be relatively unimportant in view of the minute yields of androstadienone formed from progesterone[6]. (Much larger yields (approximately 3%) of 5α-androstenone are formed from progesterone [12]).

A large number of C_{19}-steroids have been tested as possible precursors of 16-unsaturated C_{19} steroids in boar testis preparations. Testosterone and DHA [6], however, did not serve as precursors. Neither did the former steroid serve as a precursor in testis of rabbits, rats or bulls *in vitro* [45, 46]. In view of the ease of chemical dehydration of epitestosterone compared with testosterone [47], the former was considered a more likely precursor. However, when epitestosterone was incubated with testis preparations from boars[9] and from rats, rabbits and bulls[45, 46] little or no 16-unsaturated C_{19} steroids were formed.

Recently, the metabolism of testosterone has been investigated in the 105,000g supernatants of boar testis homogenate[48]. Only small yields (0·15–0·4%) of androstadienone were formed and traces of an-β (tentative identification). These results are therefore essentially in keeping with others in which testosterone was used[6]. However, when this steroid was incubated with the 105,000g supernatant of sow ovarian homogenates, good yields of androstadienone (up to 4·7% in some experiments) were obtained[48]. In the same series of experiments[48], both 16α- and 17α-hydroxyprogesterone were converted to 16-dehydroprogesterone but no 16-unsaturated C_{19} steroids were formed. Similar negative results using 16α-hydroxyprogesterone in boar testis homogenate have been obtained by other workers[49].

The search for intermediates between C_{21} steroids and 16-unsaturated C_{19} steroids

By comparison with the well-established biosynthetic pathways for C_{19}-steroids such as androstenedione and DHA, Ahmad and Gower[6] considered 17α-hydroxylated C_{21} steroids as likely intermediates, since side-chain cleavage was known to occur only after 17α-hydroxylation in androgen biosynthesis. In a double isotope experiment, however, using 17α-hydroxy-[4-^{14}C] progesterone and [7α-^{3}H] progesterone, the an-α and an-β isolated and purified contained ^{3}H as expected but no ^{14}C (Table 8). This surprising finding was subsequently confirmed and 17α-hydroxypregnenolone likewise excluded as an intermediate [9]. These results strongly suggested that, at least in boar testis, the 16-unsaturated C_{19} steroids are formed from pregnenolone or progesterone by pathways which do not involve the formation of 17α-hydroxylated derivatives before side-chain cleavage occurs. Indeed, it seems likely that such pathways may be unique in the field of steroid biochemistry. Table 8 also indicates the low yields of testosterone and androstenedione relative to those of the 16-unsaturated steroid, an-β. These results are in keeping with analytical results already described (p. 47).

Testosterone acetate is now well-known as an intermediate in the microbial conversion of progesterone to testosterone [50. 51] although it has recently been excluded as an intermediate in mammalian testicular steroidogenesis [52]. When the radioactive material was incubated with a boar testis preparation, however, it was found to be a very inefficient precursor of 16-unsaturated steroids [6]. Ahmad and Gower [6] pointed out the possibility of hydrolysis of the steroid ester to testosterone which, at that time, was already known to give rise only to traces of 16-unsaturated steroids [53]. When a specific esterase inhibitor, phenylmethylsulphonyl fluoride [54] was included in a similar incubation with testosterone acetate [20], only traces of 16-unsaturated steroids were formed and testosterone acetate was largely unhydrolysed. A parallel incubation with ^{14}C-labelled progesterone and an identical amount of the esterase inhibitor gave rise to the anticipated yields of an-α and an-β (Table 9). These experiments thus clearly excluded testosterone acetate as an intermediate in 16-unsaturated C_{19} steroid formation in boar testis.

Using unlabelled 16-dehydroprogesterone with a boar testis preparation [6], no 16-unsaturated C_{19} steroids could be detected suggesting that dehydration of progesterone did not occur prior to side-chain cleavage. Moreover, when unlabelled 5-androsten-3β-ol was used in an incubation together with radioactive pregnenolone [8] no radioactivity was trapped in the 5-androsten-3β-ol isolated even though radioactive andien-β was formed in the expected yield. Taken together, these experiments suggested that andien-β formation occurred from pregnenolone by a concerted series of reactions [8]. Fig. 6 summarizes 16-unsaturated steroid biosynthesis in boar testis *in vitro*.

The conversion of pregnenolone to andien-β

In recent years this step, or series of steps, has been studied intensively in boar testis homogenates. The enzyme or enzyme-system called 'andien-β synthetase' [55] bringing about this conversion requires NADPH and O_2 for full

Table 9. Investigation of testosterone acetate as a precursor for 16-unsaturated C_{19} steroids

Precursor	16-unsaturated C_{19} steroid formed (%)	Other products (%)
Testosterone acetate plus phenylmethylsulphonyl fluoride	None	Testosterone acetate (18·0) Testosterone (none) 5α-Androst-3-one-17β-yl-acetate (5·0)* 5α-Androst-3α- and 3β-diol-17β-yl-acetates (42·0)*
Testosterone acetate	None	Testosterone acetate (17·6) Testosterone (7·5) Androstenedione (0·38)
Progesterone plus phenylmethyl sulphonyl fluoride	An-α (2·0) An-β (12·0) 5α-Androstenone (3·0)	

Mince of boar testis were incubated for 2 h as described earlier [9]. The specific esterase inhibitor, phenylmethylsulphonyl fluoride [54], was added at a final concentration of 8×10^{-3} M.

*tentative identification only.

Yields are expressed as percentages of radioactivity incubated and are corrected for analytical losses. Reproduced from Refs. [20] and [56] by permission of J. & A. Churchill, Ltd.

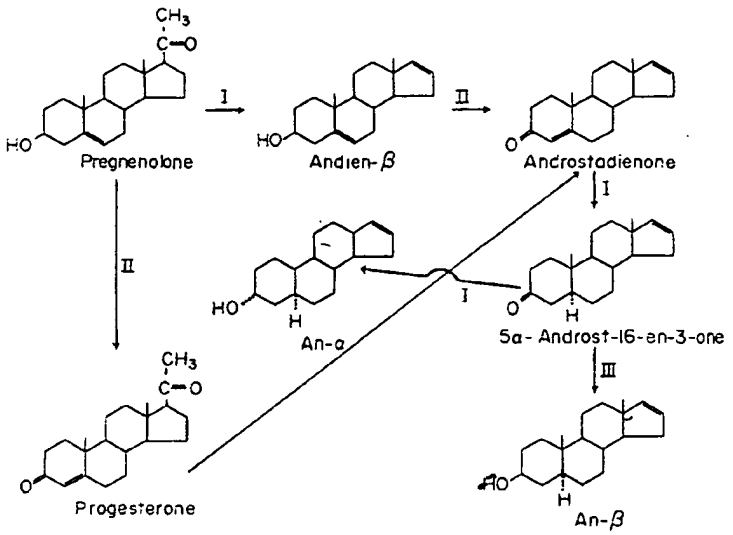

Fig. 6. Pathways of biosynthesis of 16-unsaturated C_{19} steroids in boar testis *in vitro*. I, NADPH; II, NAD^+; (based on results from Refs. 12, 156) III, NADH.

activity, NADH being a rather poor substitute [8, 56]. The 'andien-β synthetase' activity resides in the microsomal fraction of boar testis [55, 159] and enzyme activity is retained for up to three months if stored at −20°C. High yields (corrected for analytical losses) of up to 25% of andien-β have been obtained from pregnenolone in 10 min at 37°C using such preparations [55]. The rate of formation of andien-β from pregnenolone is linear up to 10 min and is also proportional to the amount of boar testis homogenate protein incubated [56, 57] (Figs. 7 and 8). Gower and

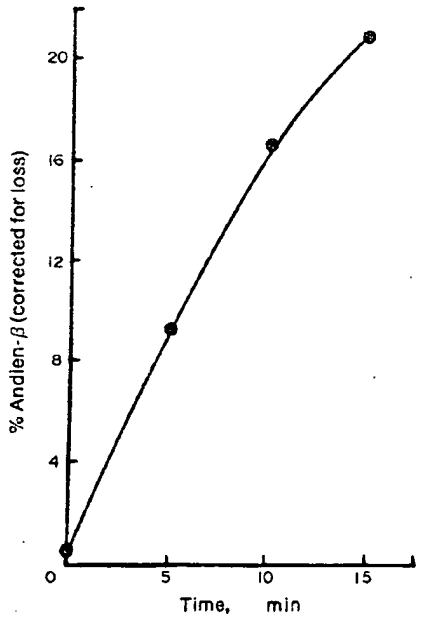

Fig. 7. Rate of formation of andien-β from [4-^{14}C] pregnenolone in boar testis homogenates. For further details see text. Data from Refs. [56] and [57].

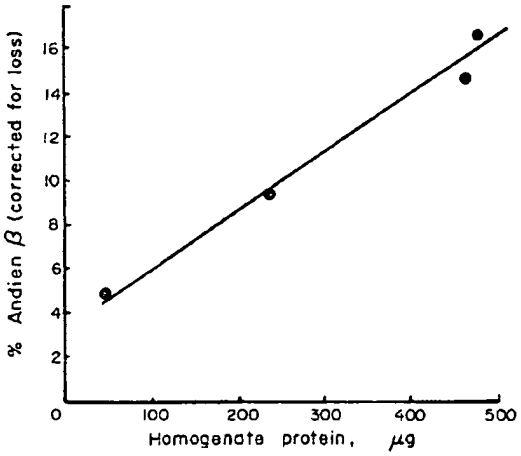

Fig. 8. Influence of the amount of boar testis homogenate incubated on the yield of andien-β formed from [4-^{14}C] pregnenolone. For further details see text. Data from Refs. [56] and [57].

Katkov [57] used as standard conditions, 200–400 μg homogenate protein incubated for 10 min at 37°C with [4-^{14}C] pregnenolone in Tris-HCl buffer (0·05M, pH 7·4) with NADPH (0·4 mM). The radioactive andien-β formed can then be readily isolated by t.l.c. first on Kieselgel G and then on $AgNO_3$-impregnated Kieselgel G, analytical losses being estimated by reverse isotope dilution (see p. 82, 86). In this way a fairly rapid assay for 'andien-β synthetase' activity in various tissues can be performed. The microsomal localisation of the enzyme [55, 159] this strongly indicates that it is distinct from the 'dehydratase' present in the 105,000g supernatant of sow ovaries and, to a lesser extent, in boar testis [48]. Moreover, the 'andien-β synthetase' is very NADPH-dependent.

Although 17α-hydroxypregnenolone has been excluded as an intermediate in this transformation [8] the possibility that dihydroxylated C_{21} steroids might be implicated was considered recently [55]. 16α,20β-Dihydroxyprogesterone had previously been used in a chemical synthesis of 16-unsaturated C_{19} steroids [49] (see p. 77) and 20β-dihydropregnenolone was found to give rise to 15–17% yields of andien-β, and the corresponding 20α-isomer only 2·7% compared with 25% for pregnenolone [55].

Short-term kinetic studies using 20β-dihydropregnenolone as substrate suggested that this steroid might be an intermediate in andien-β formation from pregnenolone and, in keeping with this, the 20β-isomer was isolated during andien-β formation [58]. Moreover, the biosynthesis of this 16-unsaturated steroid was severely inhibited (approximately 90%) if the boar testis preparation was incubated first with 20β-dihydropregnenolone before the usual incubation with pregnenolone took place. On the basis of these findings and a postulated free-radical mechanism for 16-unsaturated steroid biosynthesis [59], a reaction mechanism has been suggested [58] (Fig. 9). Using deoxycorticosterone (DOC) with boar testis preparations, Lippman and Lieberman [59] showed that a very small yield (0·046%) of androstadienone was obtained. This is very similar to the yields of this compound from progesterone [6] and it is conceivable that the 21-hydroxylase present in boar testis may bring about the conversion of progesterone to DOC and that this then forms androstadienone by a free-radical mechanism [59] (Fig.

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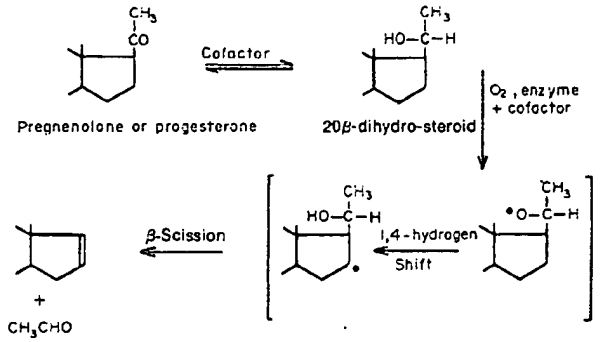

Fig. 9. Postulated free-radical mechanism for the formation of 16-unsaturated C_{19} steroids in boar testis showing the possible involvement of a 20β-dihydro-C_{21} steroid (from Ref. 58).

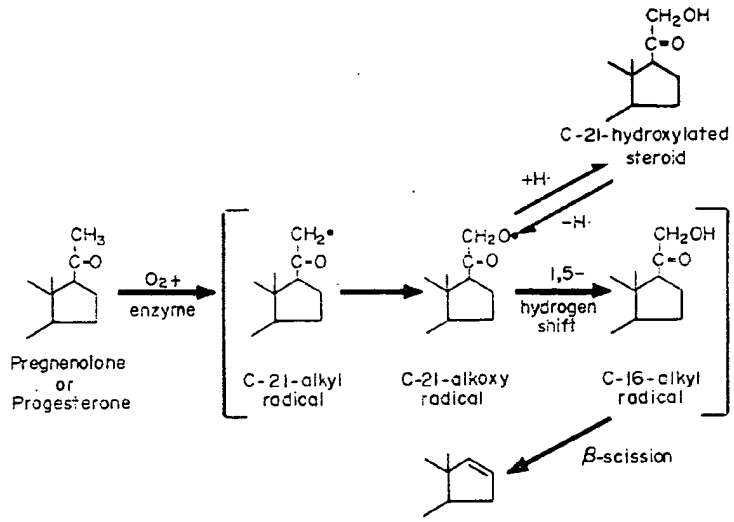

Fig. 10. Postulated free-radical mechanism for the formation of 16-unsaturated C_{19} steroids from deoxycorticosterone. Redrawn from Ref. [59] by permission of National Academy of Sciences, New York.

10). The evidence for the existence of such a sequence of reactions is based on the fact that andostadienone can be synthesized from DOC by free-radical generating reagents. such as lead tetra-acetate (see p. 77). There is a somewhat analogous situation in that, when 20α-hydroxycholesterol-3-acetate is treated with this reagent, pregnenolone is formed in good yield [60].

Following the finding of 16-unsaturated steroids in porcine submaxillary glands and in saliva, the biosynthesis of this group of steroids was studied *in vitro* [7, 57]. [4-^{14}C]Pregnenolone was converted to 3·3% of labelled andien-β (Table 10) whereas no such conversion was demonstrated in a similar preparation of submaxillary gland taken from a hog [7, 57]. This strongly suggests that this gland in the pig is under the influence of the testis. Booth [61] considers that the submaxillary gland is a secondary sex organ in the boar, since he has isolated both testosterone and 5α-dihydrotestosterone from this organ, but there is no evidence at present for the actual biosynthesis of these compounds at this site.

Compared with the testes, the adrenal cortex in the boar is an inefficient producer of 16-unsaturated steroids. An-α, an-β and androstadienone were formed from pregnenolone and from progesterone *in vitro* but only in very small quantities[6, 9] (see Table 10).

It has been shown that boar "sex odour" is reduced if the preputial gland is removed surgically[62]. Since there is a high bacterial content in this gland[63] it was considered possible for 16-unsaturated steroids to be formed either by microbial transformation or in the preputial tissue itself. However, it is now clear that the boar preputial gland is histologically similar to keratinizing epithelium with no evidence of steroid secreting cells[20] (Fig. 11). Moreover, when portions of the minced tissue and preputial fluid were incubated with [4-^{14}C] pregnenolone no ^{14}C-labelled 16-unsaturated steroids were formed[20]. These findings are in agreement with those of Patterson who found only traces of 5α-androstenone in boar preputial fluid and considers that this odour is due to phenols, notably p-cresol[64], and long-chain fatty acids[65].

Biosynthesis of 16-*unsaturated steroids in porcine testis in vivo*

When boar testes were infused *in situ* with radioisotopic pregnenolone[101] the testicular venous blood contained labelled 5α-androstenone, an-α and an-β in the ether-extractable fraction. Labelled an-α and an-β were also found in the sulphate fraction but no 16-unsaturated steroids were found conjugated as glucosiduronates, although the urine, collected during the testicular infusion, did contain radioactive an-β glucuronoside. Moreover, analysis of the infused testis revealed the presence of labelled 5α-androstenone, an-α and an-β as free steroids. These results, which are in excellent agreement with earlier work[5], on the occurrence of 16-unsaturated steroids in boar spermatic venous plasma and urine, clearly demonstrate the ability of the boar testis to synthesize these compounds.

4. BIOSYNTHESIS OF 16-UNSATURATED STEROIDS *IN VIVO*

Following the *in vitro* experiments of Dorfman and his colleagues[37, 38], testosterone was at first considered to be the precursor of androstadienone and an-α. A number of workers attempted to show that C_{19} steroids gave rise to 16-unsaturated steroids *in vivo* but without success. Testosterone and DHA[26, 66, 67] and epitestosterone[68] were all excluded as precursors of an-α and other 16-unsaturated steroids. Likewise, 16α-hydroxyprogesterone[69] did not serve as a precursor in human subjects *in vivo*. However, when [7α-^{3}H] pregnenolone and [4-^{14}C]cholesterol were administered to a woman with a virilizing adrenal adenoma[70], the urinary an-α did contain very small amounts of both ^{3}H and ^{14}C.

Recently Brooksbank and Wilson[35] have studied in detail the metabolism of a mixture of [7α-^{3}H] pregnenolone and [4-^{14}C]progesterone and a mixture of [7α-^{3}H] 4-androstene-3,17-dione and [4-^{14}C]progesterone administered intravenously to two men. In the first experiment, the value of the cumulative specific activity of the urinary ^{3}H-andien-β was sufficiently high to indicate that this compound was being derived from the circulating pregnenolone. The percentage conversion of all three precursors into urinary an-α, however, was only 0·026–0·16%, indicating that this was not formed from circulating C_{21} or C_{19} steroids but was produced in a compartment in which these steroids did not come into rapid equilibrium from the general circulation.

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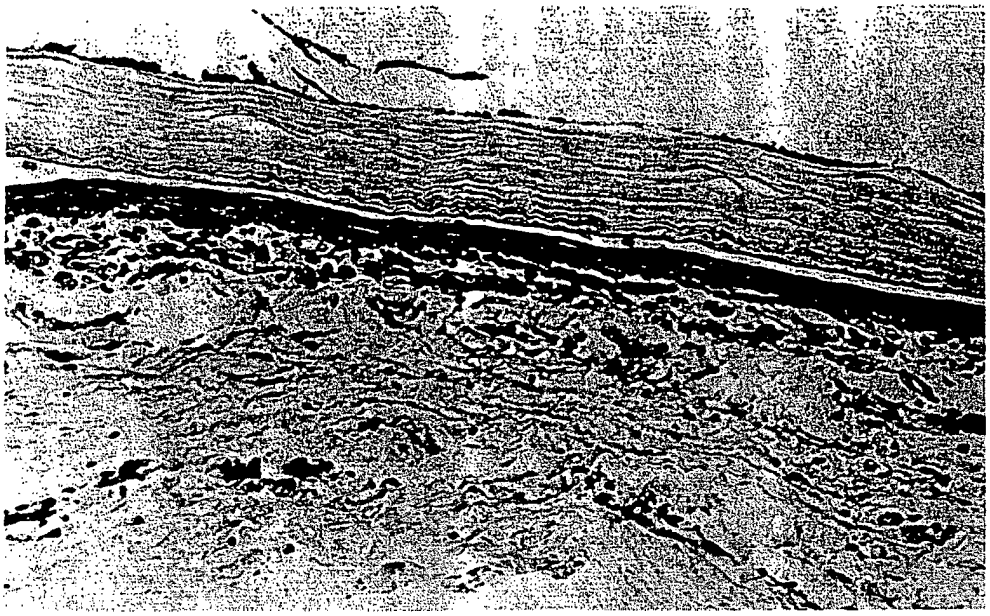

Fig. 11. Photomicrograph of a section (6μ thick) of boar preputial gland. The section was fixed in 4% formaldehyde, embedded and stained with haematoxylin and eosin. Magnification 75 × 50. Reproduced from Ref. [20] by permission of J. & A. Churchill Ltd.

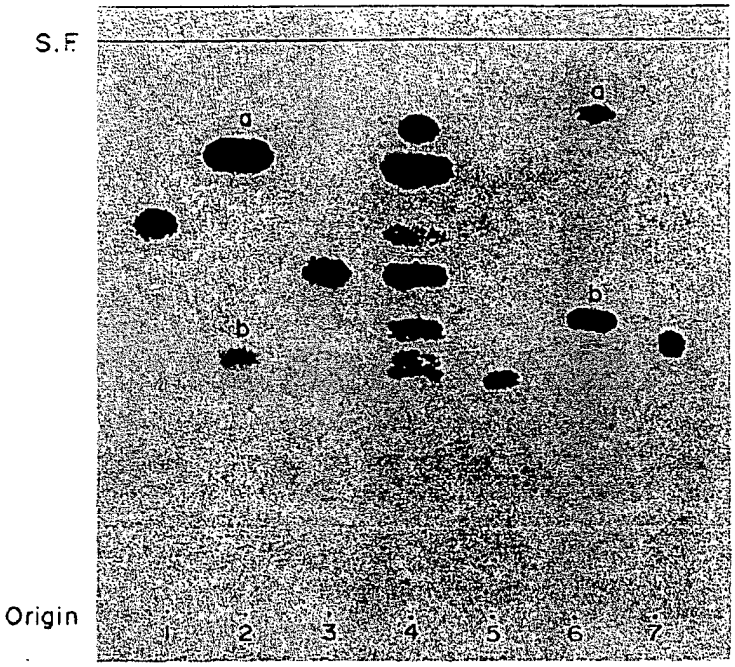

Fig. 19. T.l.c. of 16-unsaturated steroids on Kieselgel G. Lane 1, androstadienone; 2a, oestratetraenol; 2b, andien-β; 3, an-α; 4, mixture of 1, 2, 3, 5, 6 and 7; 5, an-β; 6a, 5α-androstenone; 7, 4,16-androstadien-3β-ol. The plate was run twice in benzene-ether (9:1, v/v).

(*Facing page* 64)

In keeping with these experiments showing that urinary an-α was not derived from circulating progesterone [35], urinary an-α did not increase during the pregnancies of two women although pregnanediol excretion increased as anticipated (Brooksbank and Gower, unpublished observations).

Possible sites of formation of 16-unsaturated steroids in humans

There is some evidence that andien-β is formed from pregnenolone in human testis *in vitro* [71] (see p. 69), although little or no an-α or ae-α was biosynthesized from this C_{21} steroid. Androstadienone, however, which may be the precursor of an-α and ae-α, is formed in sizeable quantities in the same incubations (Table 13).

Further experiments by Bicknell and Gower [71] indicated the testis as a source of an-α, ae-α and andien-β, or their immediate precursors, since removal of the testis resulted in a decrease in the urinary levels of these compounds (see p. 69).

As urinary an-α excretion is stimulated by administration of ACTH [24] it is conceivable that androstadienone, an-α and andien-β may be secreted by the human adrenals. Hitherto, only diseased adrenals have been studied *in vitro* and these produced no an-α from pregnenolone: androstadienone and especially andien-β were, however, biosynthesized in appreciable yields [25, 31] (Table 14).

Evidently the ovary of the sow has the ability to form androstadienone *in vitro* [48] (p. 59), but no direct evidence for the secretion of 16-unsaturated C_{19} steroids has yet been reported. Thus the origin of the major 16-unsaturated urinary steroid, an-α, still remains a mystery and further work is urgently needed to clarify the situation (see also Section 6).

5. OCCURRENCE AND BIOSYNTHESIS OF 16-UNSATURATED STEROIDS IN ENDOCRINE DISORDERS

Urinary an-α excretion has been measured in a number of women with simple hirsutism or with some abnormality of the endocrine glands. Early reports dealt with the isolation of an-α from the urine of women with adrenal tumours [73], adrenal hyperplasia [74] and luteoma of the ovary [75] but, since the authors made no attempts to estimate analytical losses, it is difficult to obtain accurate data for the urinary an-α. It seems likely, however, that there was a raised excretion of this compound in these patients. The more recent report of a woman with a virilizing adrenal adenoma [70] has already been mentioned (p. 64). In this case the urinary excretion of an-α and 17-oxosteroids was grossly elevated (Table 11).

In more recent work, urinary an-α, ae-α and andien-β have been measured, using a g.l.c. method [23] in a woman and a female infant with virilizing adrenocortical carcinomata [25, 31]. In both patients urinary an-α was grossly elevated, compared with that of normal subjects of the same age; as were the urinary ae-α, andien-β, 17-oxosteroids and testosterone (Table 11). But although there appeared to be a positive correlation in these patients between urinary 16-unsaturated steroids and androgens, no clear correlation emerged in a group of patients with simple hirsutism [23] (see Table 6).

Urinary an-α, ae-α and andien-β have also been measured in some testicular feminization (TF) patients [71]. Only in some individuals were the values raised while in others, normal amounts (for women of the same age) were being excreted (Table 12). In two patients (R.F. and S.D.) urinary an-α, ae-α and andien-β decreased after removal of the testes, indicating that these compounds or their

Table 10. Biosynthesis of 16-unsaturated C_{19} steroids in porcine tissues

Tissue preparation	Cofactor	Precursor	Yields (%) of 16-unsaturated steroids formed: an-α	an-β	androstadienone	andien-β	5α-androstenone	Reference
Testis minces	NAD^+, $NADP^+$, ATP, glucose	pregnenolone	0·9	10·0	2·3			[9]
Testis minces	as above	pregnenolone	1·6	10·6	0·06			[6]
Testis minces	as above	pregnenolone		2·0		8–15†		[8]
Testis homogenates	NAD^+, NADPH, ATP	pregnenolone	3–4†	1·9		8–15†		[7]
Testis homogenates:								
3 week old boar	NAD^+, NADPH, ATP	pregnenolone				15·5		[7]
4 week old boar	NAD^+ NADPH, ATP	pregnenolone				8·6		[7]
8 week old boar	NAD^+, NADPH, ATP	pregnenolone				8·5		[7]
Testis microsomes	NADPH	pregnenolone				25·6		[55, 58]
		20α-dihydro-pregnenolone				16·0		[55, 58]
		20α-dihydro-pregnenolone				2·7		[55, 58]
Testis minces	as Ref. [9]	progesterone	1·5–1·8†	10·0–13·0†	0·006–0·008†			[6]
Testis homogenate	NAD^+, NADPH, ATP, glucose	progesterone					3·6	[12]
Testis minces	as Ref. [9]	progesterone	2·0	12·0			3·0	[20]
Testis minces	as Ref. [9]	progesterone		>10% of 16-unsaturated C_{19} steroids				[49]
Testis mince	NAD^+, NADPH, ATP	progesterone		7·0		nil		[8]
Testis homogenate	NADPH	17α-hydroxy-pregnenolone			none formed			[8]
Testis mince	as Ref. [9]	17α-hydroxy-progesterone			none formed			[6]
Testis mince	as above	16α-hydroxy-progesterone			none formed			[49]
Testis mince	as above	16-dehydro-progesterone			none formed			[6]

Testis mince	as Ref. [9]	pregnenolone			none formed	[8]
		17α-hydroxy-progesterone			none formed	[6]
Testis mince	as above	16α-hydroxy-progesterone			none formed	[49]
Testis mince	as above	16-dehydro-progesterone			none formed	[6]
Testis homogenate		deoxycorticosterone			0·046	[59]
Testis minces	as Ref. [9]	testosterone			none formed	[6, 53]
Testis (105,000 *g* supernatant)	NADPH-generating system	testosterone		trace*	0·15–0·40	[48]
Testis homogenate	NAD^+, NADPH, ATP	epitestosterone		< 1·0 of androstadienone, andien-β and an-β		[8]
Testis minces	as Ref. [9]	testosterone acetate	nil	0·01	0·01	[6]
Testis minces	as above	testosterone acetate			none formed	[20]
	plus phenylmethyl sulphonyl fluoride	testosterone acetate			none formed	[20]
Testis minces	as Ref. [9]	DHA	nil	0·01	0·01	[6]
Adrenocortical minces	as Ref. [9]	pregnenolone	0·03	0·4	0·08	[9]
		progesterone	0·007	0·015	0·04	[6]
		testosterone	nil	nil	nil	[6]
Ovary (no corpora lutea) 105,000 *g* supernatant	NADPH-generating system	testosterone	trace*		0·05–4·7	[48]
Submaxillary gland homogenate	NADPH	pregnenolone			3·3	[7]
Submaxillary gland homogenate (male castrate)	NADPH	pregnenolone			nil	[7]
Preputial gland plus fluid (homogenate)	NAD^+, NADPH, ATP glucose	pregnenolone		none formed		[20]

*tentative identification.
†several experiments.

Table 11. Urinary 16-unsaturated C_{19} steroids and 17-oxosteroids (mg/24 h) in cases of adrenal adenoma and carcinoma

Patient	Clinical condition	Age (yr)	an-α	ae-α	andien-β	andro	aetio	DHA	Reference
R.C.	Adenoma with Cushings' syndrome (hirsute)	29	0·050	—	—	—	—	—	[23]
	Post-operatively (14 days)		0·006	—	—	—	—	—	[23]
S.W.	Virilizing carcinoma	31	2·06 (G 1·46; S 0·6)	0·10	0·157	19·0 (G 13; S 6)	42·5 (G 23·5; S 19)	123·5 (G 46·3; S 77·2)	[23, 31]
	Post-operatively (1 month)		0·11	—	—	0·11	4·0	0·13	
V.D.	Virilizing carcinoma	14	0·51	0·004	0·026	2·6 (G 1·3; S 1·3)	1·6 (G 1·4; S 0·2)	21·6 (G 7·5; S 14·1)	[23, 25]
	Post-operatively (14 months)		0·014	N.D.	N.D.	0·03	0·06	0·01	
—	Virilizing adenoma		20‡	—	—	17·0‡	82·0‡	51·0‡	[70]
Mean normal values for adult women (with ranges)			0·36* (0·23–0·42)	0·0048*	0·0207*	1·4† (0·9–2·6)	1·8† (0·6–2·2)	0·56† (0·2–1·1)	
Mean normal values for infants			N.D.*	N.D.*	N.D.*	0·1	0·1	0·1	

*Ref. [23].
†Ref. [31] (by permission of Periodica, Copenhagen).
All values were obtained by the g.l.c. method of Brooksbank and Gower[23] except for those marked which‡ were obtained as weights of isolated crystalline material. G = glucuronoside, S = sulphate, Andro = androsterone; aetio = aetiocholanolone. N.D. means not detected.

Table 12.

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Urinary An-α, mg/g Creatinine

Fig. 12. E

Table 12. Urinary excretion of 16-unsaturated C_{19} steroids (μg/g creatinine) in testicular feminization

Patient	an-α	ae-α	andien-β
L.R. (pre-operatively)	209 (130–360)*	6·9 (3·0–10·4)*	29·0 (12·5–46·0)*
R.F. (pre-operatively)	1280	11·7	59·0
R.F. (post-operatively)	463	6·3	21·0
S.D. (pre-operatively)	631	1·83	9·7
S.D. (post-operatively two days)	525	0·52	2·5
S.D. (post-operatively seven days)	146	0·0	—
F.N.† (pre-operatively)	339	5·7	25·0
Normal values	230–420‡	4·8‡	20·7‡

*Mean and range of several estimations.
†Male pseudohermaphrodite with histologically normal testes.
‡From Ref. [23]; data from Refs. [71] and [106].

immediate precursor(s) had their origin in these glands. The fact that administration of HCG to another TF patient resulted in 2–4 fold increases in 16-unsaturated steroid excretion[71] (Figs. 12, 13) gave further support to this suggestion; normally the HCG effect occurs only in men. Administration of ACTH also caused an increase in urinary an-α in normal men and women[22, 24] as well as in an-α, ae-α and andien-β in TF syndrome (Figs. 12, 13) thus suggesting an adrenal origin for these compounds[71].

Bicknell and Gower[71], however, were unable to show the formation of more than traces of an-α and ae-α from pregnenolone or progesterone when testis minces from TF patients were incubated *in vitro* (see Table 13); andien-β was formed from pregnenolone in yields of 1–8% but, in contrast to porcine testis (Ref. 6, Table 8), approximately six times as much androstadienone was formed from progesterone than from pregnenolone. This finding in testis tissue from three

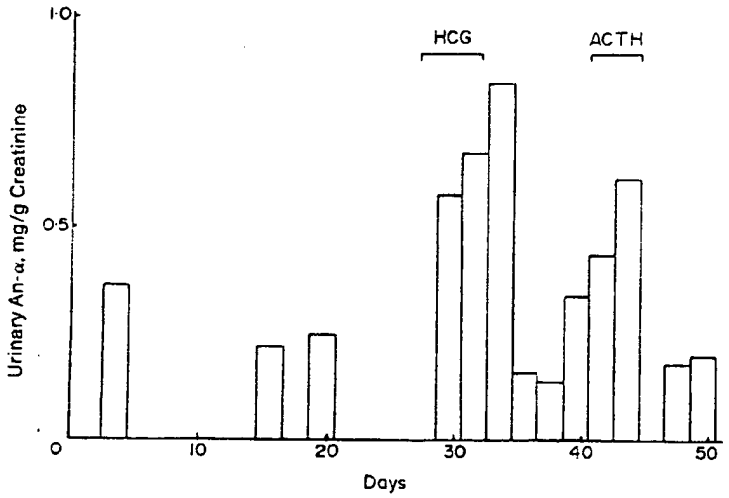


Fig. 12. Effect of ACTH and HCG administration on urinary an-α in a woman (L.R.) with testicular feminization (from Refs. 71 and 106).

Table 13. Biosynthesis of 16-unsaturated C_{19} steroids in human testicular feminization testis preparations

Patient	Precursor	Percentage yields (corrected for analytical losses) Androstadienone	andien-β	an-α†	ae-α†	Ratio of: androstadienone from pregnenolone / androstadienone from progesterone
R.F.	Pregnenolone	0·22	5·1	0·1	nil	0·25
	Progesterone	0·86	nil	0·1	0·2‡	
J.M.	Pregnenolone	<0·1	1·0	nil	nil	<0·5
	Progesterone	0·2	nil	nil	0·2‡	
S.D.	Pregnenolone	0·48	8·54			
	Progesterone	1·33	nil			0·36
	Testosterone	nil	nil			
F.N.*	Pregnenolone	2·8	4·2	—	—	
	Progesterone	0·24	nil	—	—	10·2
	Testosterone	trace	nil	nil	nil	

*Patient F.N. was a male pseudohermaphrodite with histologically-normal testes.
†Tentative identification only.
‡Approximate values only.
Data from Refs. [71] and [106].

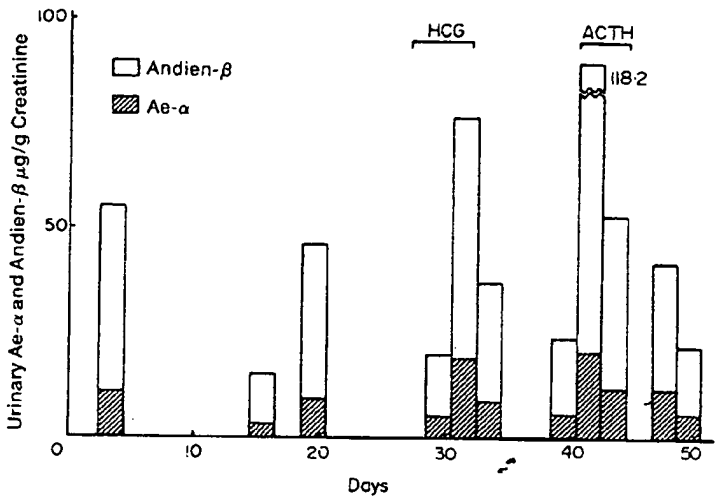


Fig. 13. Effect of ACTH and HCG administration on urinary ae-α and andien-β in a woman (L.R.) with testicular feminization (from Refs. 71 and 106).

TF patients[71] also contrasts directly with results obtained using histologically normal testis from a male pseudohermaphrodite[71]. In this case andien-β (4%) was formed from pregnenolone but considerably more androstadienone (2%) was formed from this precursor than from progesterone (Table 13). The significance, if any, of these differences in 16-unsaturated C_{19} steroid biosynthesis has yet to be determined. Tentative evidence for the formation of andien-β and androstadienone from pregnenolone in TF tissue has been presented in earlier work[76].

Some evidence has been obtained from the biosynthesis of 16-unsaturated steroids in human adrenal carcinoma tissue; pregnenolone and progesterone were metabolised to andien-β and androstadienone but C_{19} steroids such as testosterone or DHA did not serve as precursors[25, 31] (Table 14) thus confirming earlier experiments performed with porcine testis[6].

Only two reports have so far appeared concerning 16-unsaturated steroids in human ovarian tissue. After incubating human follicles with radioactive pregnenolone[76], fast-running metabolites were noted when the tissue extract was subjected to paper chromatography. Subsequent thin-layer chromatography of this material, using 16-unsaturated steroids as markers, resulted in the tentative identification of andien-β and androstadienone. Similar results have been obtained using polycystic ovarian tissue with labelled pregnenolone[77].

Table 14. Yields (as percentage of radioactivity incorporated/g wet wt. of tissue) of 16-unsaturated C_{19} steroids formed in minces of adrenal carcinoma

Precursor	andien-β	androstadienone	an-α	an-β	Reference
Pregnenolone	0·2	N.D.	N.D.	N.D.	[31]
Pregnenolone	4·7	N.D.	N.D.	N.D.	[25]
Progesterone	N.D.	<0·03	N.D.	N.D.	[31]
Progesterone	0·15	N.D.	N.D.	N.D.	[25]
Testosterone	N.D.	N.D.	N.D.	N.D.	
DHA	N.D.	N.D.	N.D.	N.D.	

N.D. not detected.

6. FURTHER METABOLISM OF 16-UNSATURATED C_{19} STEROIDS

(a) *In vitro.* The metabolism of ^{14}C-labelled andien-β, prepared biosynthetically from [4-^{14}C]pregnenolone in a boar testis homogenate [8], has been studied. Andien-β is converted in high yield (31%) to androstadienone when incubated with boar testis homogenate and NAD^+ (0·4 mM) but if both NADPH and NAD^+ are added as cofactors, there is a relatively small yield (4%) of an-β. Recently, 5α-androstenone, labelled with tritium, was shown to be converted to an-α (and an-β) in preparations of porcine parotid gland [4]. Such evidence is in keeping with the relatively large amounts of, particularly, an-α that have been isolated from this source in pigs (see Table 1), and explains the fact that the alcohol occurs in larger quantities than the ketone [3, 4, 17].

(b) *In vivo.* When isotopically-labelled androstadienone became available through a chemical synthesis and purification [78], its metabolism was studied [30, 33] after intravenous administration to two healthy men and one healthy woman (in the follicular phase). The disappearance curves for the purified plasma androstadienone approximated to those of a two-pool distribution [33] and the estimated plasma production rates (PPR) (μg/24 h) were 975 and 1341 in the two men and 456 in the woman. The calculated urinary production rates (UPR) (μg/24 h) of the androstadienone in the men, calculated from the cumulative specific activity of the urinary radioactive an-α were, however, very much higher than the corresponding PPR's and indicate that the urinary an-α does not arise entirely from the circulating androstadienone in men; in the woman the discrepancy between estimated PPR and UPR was very much less. Subsequent investigations of urinary 16-unsaturated steroids after intravenous administration of [^{14}C]-androstadienone to a man and a woman [30] indicated that 95% of the ^{14}C was in the glucuronoside and only 5% in the sulphate fraction. An-α accounted for 79% (in the man) and 51% (in the woman) of the radioactivity of the glucuronoside fraction while some activity was found in ae-α and also in an-β, a compound not previously detected in human urine [29] although present in boar urine [5].

Further analyses [30] of the urines obtained have revealed the presence of polar metabolites including $C_{19}O_3$ compounds that are neither phenolic nor acidic and that behave like androstanetriols chromatographically (B. W. L. Brooksbank, personal communication). These metabolites were formed to a much greater extent in the woman than in the man [30]. Such a metabolic route would be similar to that for oestratetraenol which is known to give rise to a 16,17-glycol [72] (see p. 73). If polar metabolites are also formed from an-α, this might explain the very low recoveries (1·5–11%) of this steroid in urine after an-α was administered to two men [24]. Moreover, the extra an-α did not appear in the urine until the second day after the injection. Such findings are in keeping with a fairly extensive metabolism of an-α rather than this being an end-product of metabolism of other steroids.

7. BIOSYNTHESIS AND FURTHER METABOLISM OF OESTRATETRAENOL

The C_{18} steroid oestratetraenol has weak oestrogenic activity (see p. 97) and is implicated in oestrogen metabolism. As mentioned earlier (p. 56) it occurs in human pregnancy urine [36]. Knuppen and Breuer [79] were the first to show that testosterone could be converted to androstadienone in a human placental microsomal preparation and that this compound was further metabolised to the phenolic 16-unsaturated steroid, oestratetraenol. When the latter was administered to

human subjects intravenously, an increase in the urinary "oestriol" fraction was noticed[80] and a more detailed investigation showed that the oestrogen concerned was 16-epioestriol[81]. The conversion of oestratetraenol to epioestriol may be via the 16,17-epoxide as an intermediate, the epoxide ring subsequently opening to form the 16β,17β-glycol[72] (Fig. 14).

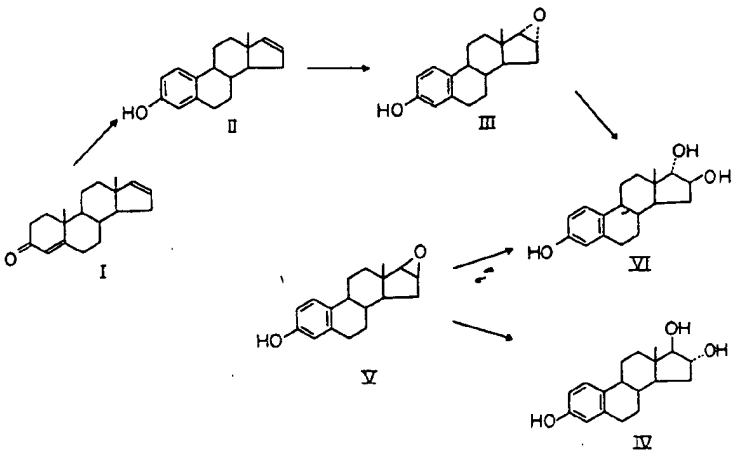
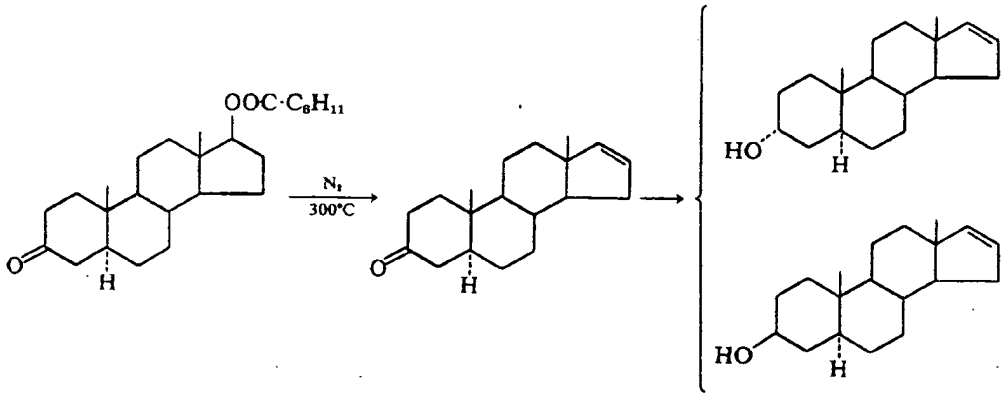


Fig. 14. Biosynthesis and metabolism of oestratetraenol. I, androstadienone; II, oestratetraenol; III, 16α,17α-epoxyoestratrien-3-ol; IV, oestriol; V, 16β,17β-epoxyoestratrien-3-ol; VI, epioestriol.

8. CHEMICAL SYNTHESIS OF 16-UNSATURATED STEROIDS

Many of the methods for the synthesis of this group of compounds have been reviewed recently[78].

In early experiments[82] it was shown that the hexahydrobenzoate of 17β-hydroxy-5α-androstan-3-one (I) could be converted to 5α-androstenone by dry-distillation at 300°C and that a mixture of an-α and an-β was formed from this by reduction:

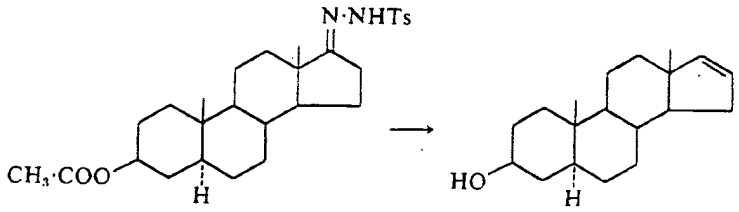


Using the same method of dry-distillation, Fajkos[83] obtained an oil from the hexahydrobenzoate of I which, after saponification, resulted in 5α-androstenone (yield 28%); $LiAlH_4$ reduction then afforded an-β in 78% yield. The Prelog group[84] also showed that the benzoate of 17β-hydroxy-5β-androstan-3-one could be converted by dry-distillation to 5β-androstenone, the latter being reduced

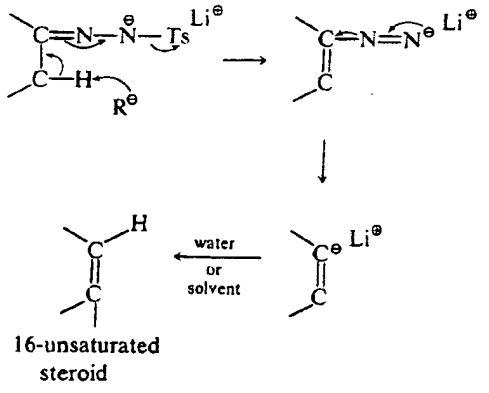
subsequently to a mixture of ae-α and 5β-androst-16-en-3β-ol. Epitestosterone benzoate, under the same conditions, was converted to androstadienone [84]. The dehydration of 17β-xanthates [85] and of 17β-p-toluene sulphonates (tosylates) (cited in Ref. 37) also results in the formation of 16-unsaturated steroids.

Androstadienone is formed in another reaction involving testosterone in which the latter, in pyridine solution, is treated with bromoacetamide [86]. Sulphur dioxide gas is then added until no further reaction to starch-iodide paper is obtained. Extraction and purification is said to yield androstadienone, although later workers [78] were unable to confirm this. In 1962 a study was published [87] of the use of aprotic solvents for nucleophilic substitution at C-3 and C-17 since, under these conditions, structural rearrangements through migration of the C-18 methyl group are inhibited. Thus, when testosterone tosylate was subjected to high temperature acetolysis in N-methyl pyrrolidone with tetra-butyl ammonium acetate at 160°C, the products were androstadienone (57%) and 17α-testosterone acetate (34%). The proportions of these products, however, vary when the reaction is scaled down (see below).

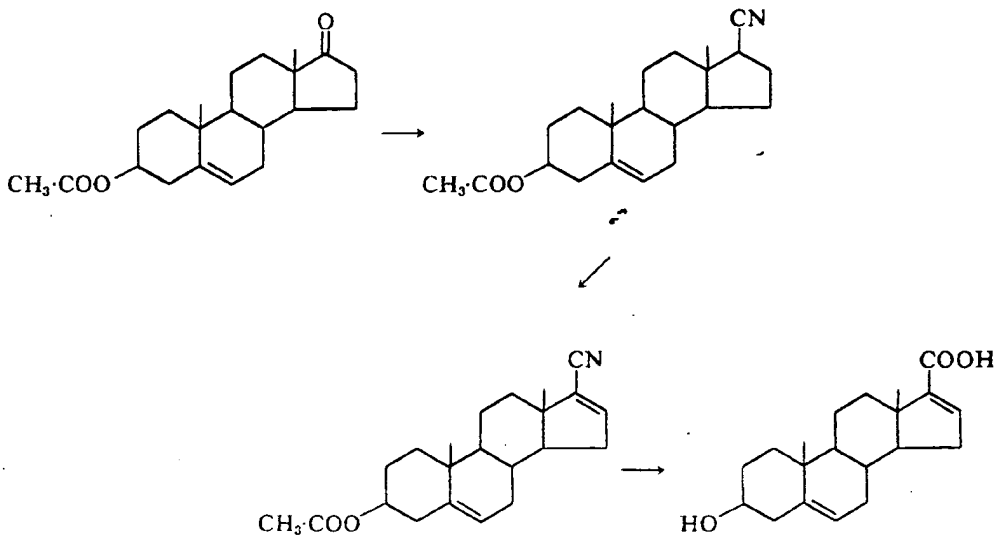
Caglioti and Magi [88] investigated the behaviour of tosylhydrazones of 17-oxosteroids when treated with $LiAlH_4$ in tetrahydrofuran (overnight refluxing). Under these conditions the tosylhydrazone of 3β-acetoxy-5α-androstan-17-one was converted to an-β in 70% yield:



The same derivatives, dissolved in dry ether, also form the corresponding olefins when treated with greater than two equivalents of alkyl lithium compounds at room temperature [89]. The reaction proceeds smoothly even at −20°C and this fact could be particularly useful for volatile or sensitive olefins. A carbanion intermediate is proposed as follows:



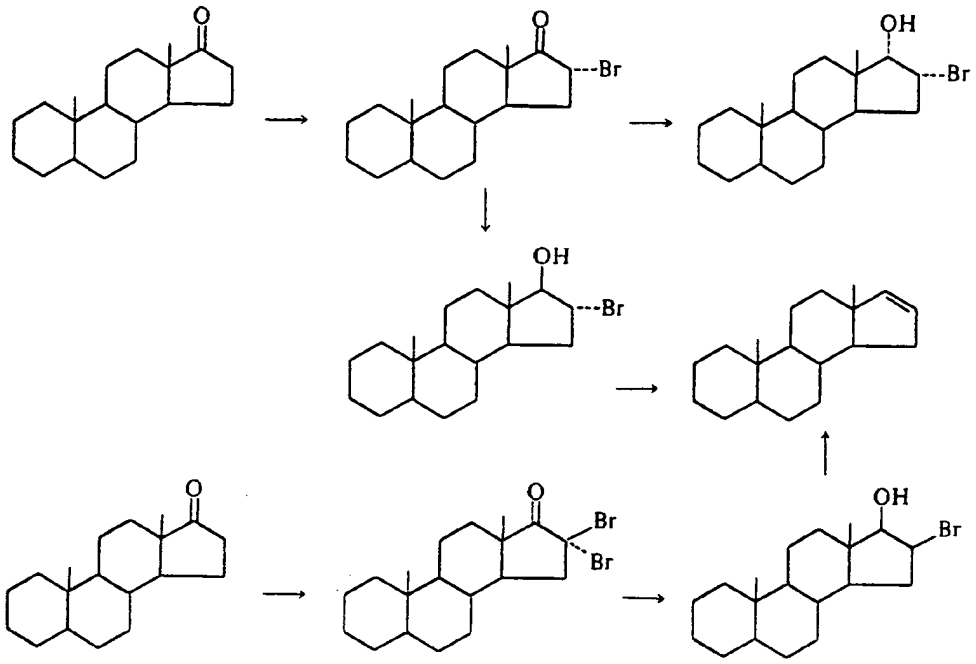
A four-step synthesis of 16-unsaturated steroids has been reported[90] that involves the synthesis of 3β-hydroxy-5,16-androstadien-17β-carboxylic acid from DHA acetate[91]. This was subsequently converted to the cyanohydrin which, on heating with $POCl_3$ in pyridine, resulted in 17β-cyano-5,16-androstadien-3β-yl-acetate (m.p. 210°). Vigorous alkaline hydrolysis afforded 3β-hydroxy-5,16-androstadien-17β-carboxylic acid:—



The unsaturated acid was subsequently refluxed in quinoline in the presence of activated copper chromite as catalyst when andien-β was formed. A similar series of reactions was employed later[21] for the synthesis of an-α starting with androsterone acetate. After 17-cyanohydrin formation, dehydration at C-17 was achieved by heating with $POCl_3$ under pressure to give 17β-cyano-5α-androst-16-en-3α-yl-acetate in 58% yield. Vigorous alkaline hydrolysis under pressure at 180–185° for 5 h resulted in 3α-hydroxy-16-aetioenic acid (79·6% yield). The final step involved decarboxylation in boiling quinoline in the presence of copper chromite [cf. 90] and the crude an-α so obtained was purified by sublimation, the overall yield being approximately 20% without recycling of intermediates.

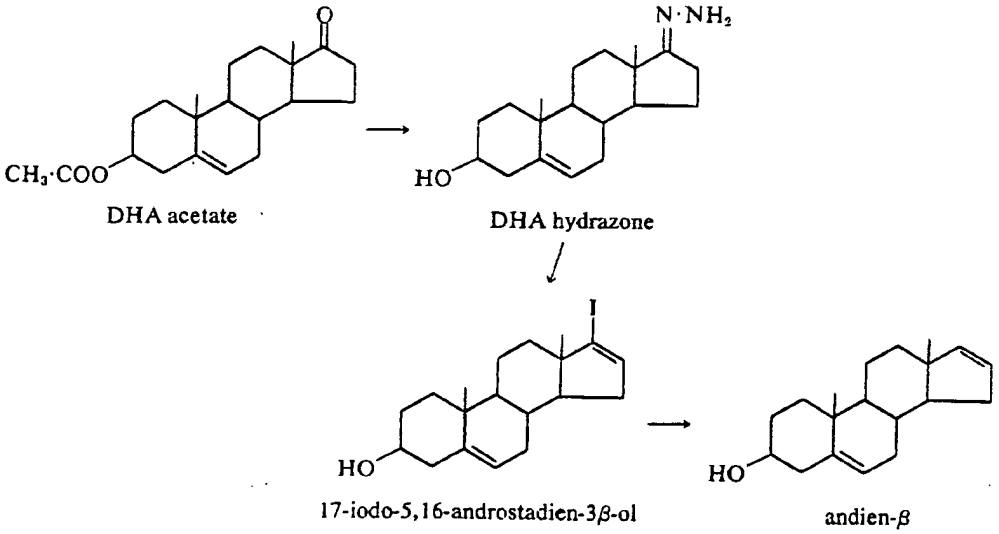
The synthesis of andien-β has recently been described[59] using the methods outlined above[90, 91] except that hydrolysis of the 17-cyano-3-acetoxy-5,16-androstadien-3β-yl-acetate was achieved by refluxing in glycerol in the presence of KOH at 175° for 20 h[92].

The synthesis of the hydrocarbon, 16-androstene, has been described by Shoppee and his collaborators[93] who used androstan-17-one as starting material. Bromination in acetic acid at 15°C resulted in 16α-bromo androstan-17-one and this, on reduction, gave either the 16α-bromo-17α-hydrin ($NaBH_4$ in methanol at 20°C) or the 16α-bromo-17β-hydrin ($LiAlH_4$ in ether at 0°C). A third epimeric bromohydrin was formed by dibromination of androstan-17-one with bromine in ether-acetic acid solution at 36°C, followed by reduction ($NaBH_4$ in methanol at 15°C) giving the 16β-bromo-17β-hydrin:



All three epimeric bromohydrins could be converted to 16-androstene by brief treatment with zinc in acetic acid.

A three-step synthesis of andien-β, also involving a 17-oxosteroid as starting material, was described in 1962[94]. DHA acetate, with hydrazine and triethylamine as catalyst, gave the hydrazone. Oxidation was then achieved using iodine in triethylamine-tetrahydrofuran, resulting in the vinyl iodide. Finally, reduction with sodium and ethanol afforded andien-β:



DHA acetate

DHA hydrazone

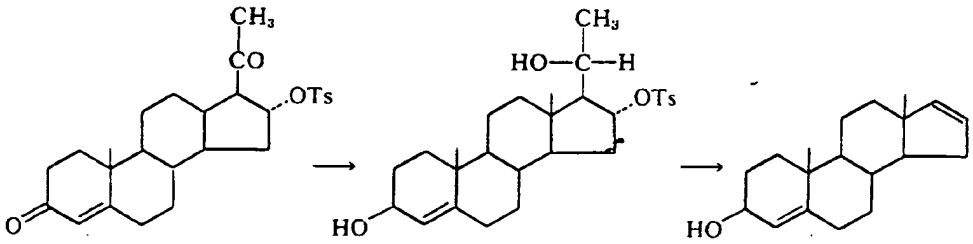
17-iodo-5,16-androstadien-3β-ol

andien-β

Using another 17-oxosteroid, epiandrosterone, as starting material, the corresponding 16-unsaturated steroid (an-β) has been obtained by a hydroboration method[95]. The 3β-acetate of epiandrosterone was converted to 17,17-diethoxy-5α-androstan-3β-yl-acetate(I) by treatment with ethyl formate in the

presence of one drop of concentrated sulphuric acid. On heating compound I under N_2, 3β-acetoxy-17-ethoxy-5α-androst-16-en-3β-yt-acetate was formed and this was converted to an-β on treatment with diborane, followed by refluxing with acetic anhydride.

Recently [49] a detailed study has been made of the heterolytic fragmentation of pregnane-16,20-diols and their mesylates, tosylates and sulphates. For example, the tosylate of 16α-hydroxyprogesterone was reduced with $NaBH_4$ to 3β,16α, 20β-trihydroxy-4-pregnene-16-tosylate. On refluxing with potassium t-butoxide in t-butanol, 4,16-androstadien-3β-ol was formed in 29% yield:



The same 16-unsaturated steroid was formed, together with other products, starting with the mesylate of 16α-hydroxyprogesterone. In further experiments [49] it was shown that, when the 16-mesylates of 3β,16β,20β-trihydroxy-5α-pregnan-3β-acetate and 3β,16α,20α-trihydroxy-5α-pregnan-3β-acetate were subjected separately to refluxing with potassium t-butoxide (as described above), among the products were small quantities of an-α (< 1%) (from the 20β-epimer) and an-β (2%).

In a recent paper, Lippman and Lieberman [59] have described the synthesis of androstadienone from deoxycorticosterone (DOC) using the free-radical generating reagent, lead tetra-acetate. Some 4-androsten-3-one was also formed in the reaction. Androstadienone was also formed from DOC using a variety of other hydrogen-abstracting reagents, such as cumene- and t-butyl-hydroperoxides, phenyl azotriphenylmethane, or by photolysis of the C-21 nitrite that was prepared from DOC using NOCl. The implication of this reaction in the biosynthesis of androstadienone and the possible mechanism are discussed in Section 3, page 62.

Chemical synthesis of oestratetraenol

This phenolic 16-unsaturated steroid can be obtained by three of the methods described above:

(i) Dry-distillation of 17β-oestradiol dibenzoate at 300–310°C under water-pump vacuum results in oestratetraenol 3-benzoate [96]. The 16-unsaturated phenol (m.p. 127–129°) can readily be obtained from the benzoate and is almost odourless but has a slight musky smell when warmed.

(ii) Huffman and co-workers [97] selectively benzoylated oestradiol-3,16β to give the 3-benzoate. On refluxing the C-16 tosylate in collidine, oestratetraenol 3-benzoate was formed in 44% yield. These workers discovered that oestratetraenol passes preferentially into the organic phase when partitioned between benzene and 0·1 N NaOH and this property has proved especially useful in the purification of oestratetraenol from uncleaved diol.

(iii) By refluxing the tosylhydrazone of oestrone 3-methyl ether overnight with $LiAlH_4$ in dry tetrahydrofuran, 3-methoxy oestratetraenol was formed in 60-70% yield [88]. A particular feature of this reaction is that no migration of the C-18 methyl group occurs under the conditions used.

Preparation of radioactive 16-unsaturated steroids

Recently some of the available methods for the synthesis of androstadienone were surveyed [78] since this steroid was required by the authors to be labelled with ^{14}C and with tritium. The high temperature acetolysis method from testosterone [87] appeared to be suitable but, on scaling-down the quantities of reagents in a pilot experiment, it was found that the yield of androstadienone was only 38% under very carefully controlled conditions instead of 57% quoted in Ref. [87]. The crystals obtained slowly underwent decomposition and three more recrystallizations were required to give a product that did not subsequently decompose (overall yield approximately 6%). Further problems arose when ^{14}C-labelled and ^{3}H-labelled testosterone were used as starting materials, in that the labelled androstadienone obtained was heavily contaminated with at least three impurities that may have been ring D isomers of androstadienone. The latter was purified using column chromatography on $AgNO_3$-impregnated silicic acid (Fig. 15) and then on alumina (see p. 79) giving a yield of approximately 6% for the pure compound.

Synthesis of 16-unsaturated steroid conjugates

The sulphates of this series of compounds do not seem to have been prepared but presumably the available methods for other steroid sulphates could be employed [98–100].

The glucuronoside of an-α has been synthesized [21] by treating an-α, dissolved in benzene and in presence of Ag_2CO_3, with methyl α-bromotri-O-acetyl glucuronate dropwise over a period of 1·5 h during which time the benzene was continuously distilled. On filtering and evaporating, methyl (androst-16-en-3α-yl-2:3:4-tri-O-acetyl-β-D-glucopyranosid)uronate was obtained in 27% yield. By treating with $Ba(OH)_2$ followed by precipitation of $BaSO_4$ with H_2SO_4, androst-16-en-3α-yl-β-glucosiduronic acid was obtained in 56% yield. This conjugate was employed [21] to estimate losses during the hydrolysis of an-α glucuronoside from human urine.

9. CHROMATOGRAPHIC SEPARATION OF 16-UNSATURATED C_{19} STEROIDS

(a) *Column chromatography*. Because of the extremely low polarity of this group of steroids, alumina chromatography has been utilised to great advantage in their isolation provided that low polarity solvents are used for elution. Thus, early workers in this field were able to separate 16-unsaturated steroids from more polar compounds such as DHA and testosterone (e.g. Ref. [1]). Using a single eluting solvent mixture, benzene–light petroleum (1 : 1, v/v), Brooksbank and his colleagues [21], were able to elute an-α from urine extracts and the column fractions so obtained were sufficiently 'clean' for colorimetric or g.l.c. estimation to be carried out [21, 23, 29]. Even for the estimation of the minor urinary 16-unsaturated steroids, ae-α and andien-β, the eluted fractions were suitable for quantitative analysis by g.l.c. without the need for a prior t.l.c. step [23]. Using alumina, partially de-activated with water (4–5%, v/w), Brooksbank and Gower

Fig. 1
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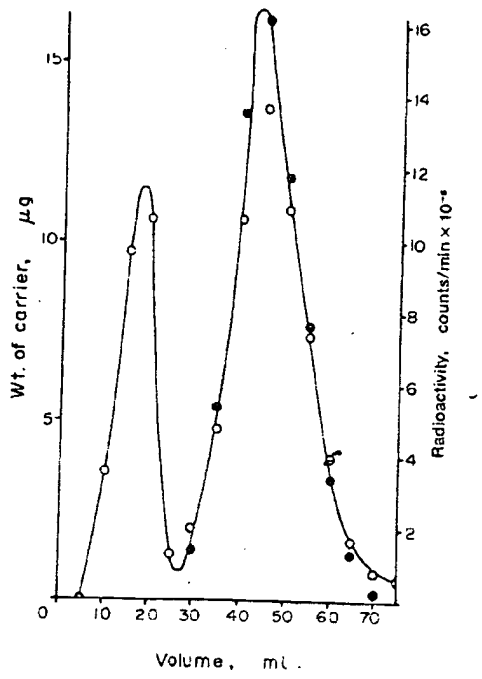

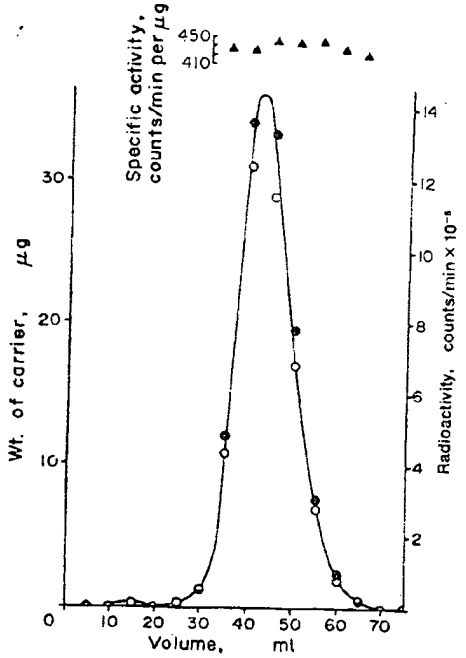

Fig. 15. Purification of synthetic [7α-^{3}H]androstadienone[78] by column chromatography on (a) $AgNO_3$-impregnated silicic acid and (b) Al_2O_3, partially deactivated with water (4–5%, v/w)[9]. The weight (μg) of carrier andostradienone (●) was determined by g.l.c. and radioactivity (○) by liquid scintillation counting. ▲, specific radioactivity (cpm/μg × 10^{-3}). (a) from Gower (unpublished) and (b) redrawn from Ref. [78] by permission. Counting efficiency 41%.

and their colleagues have shown the following approximate pattern of elution with benzene–light petroleum (1:1, v/v): 30–90 ml contains an-α and androstadienone; 90–110 ml contains ae-α; 110–175 ml contains andien-β and an-β. More polar compounds, such as androsterone, DHA and testosterone, can then be eluted from the same column using benzene containing varying proportions of ethanol (Table 15). This technique has been utilised for the separation of 16-unsaturated steroids and 17-oxosteroids in urine extracts [5, 23, 25, 31]. Similar columns have been used extensively by Gower and his colleagues [6, 9, 41–43, 55] in the separation of labelled 16-unsaturated steroids formed in incubations of tissues from various species (Fig. 16). The specific activity of the very non-polar compound 5α-androstenone (labelled with ^{14}C) was determined using an alumina column and benzene–light petroleum (1:9, v/v) as eluent [12]. This very non-polar solvent mixture has been utilised recently [101] to separate fat from 16-unsaturated C_{19} steroids in extracts obtained from boar testis and testicular vein plasma. Using partially deactivated alumina, fat was eluted first with benzene–light petroleum (1:9, v/v) followed by 5α-androstenone. An-α and an-β could be eluted next by increasing the percentage of benzene in the eluent mixture to 50% (as above). In this way, evidence has been obtained for the presence in boar testicular vein plasma of radioactive an-α, an-β and 5α-androstenone biosynthesized *in vivo* through a testicular infusion of isotopically-labelled pregnenolone [101]. These preliminary findings are in accord with the analytical and *in vitro* results already described (Sections 2 and 3).

Column chromatography on silicic acid has also been employed for the separation of mixtures of fat and 16-unsaturated steroids, extracted from porcine peripheral plasma, testes and salivary glands [4]. Fat was eluted first using cyclohexane followed by 5α-androstenone using more polar solvents (Fig. 17). An-α and an-β could be eluted from similar columns with mixtures of cyclohexane and ethyl acetate. It has not been possible to separate andien-β and an-β by alumina column chromatography even though gradient elution has been attempted (Gower,

Table 15. Elution of 16-unsaturated C_{19} steroids from alumina*

Steroids eluted	Eluent	Volume of eluent (ml)‡
5α-androstenone	benzene–light petroleum† (1:9 v/v)	50–100
androstadienone, an-α	benzene–light petroleum (1:1 v/v)	30–90
ae-α	benzene–light petroleum (1:1 v/v)	90–110
andien-β, an-β, cholesterol	benzene–light petroleum (1:1 v/v)	110–175
progesterone, androstenedione, androsterone, pregnenolone	benzene containing ethanol (0·2% v/v)	50
pregnenolone, testosterone, aetiocholanolone, DHA	benzene containing ethanol (0·5% v/v)	50

*Alumina (type H, 100–200 mesh; Peter Spence and Co. Ltd., Widnes, Lancs.) was partially deactivated by the addition of water (4–5% v/w). 5g was used in these experiments.

†Light petroleum (b.p. 80–100°C) was treated with conc. H_2SO_4, washed and dried [9].

‡Volumes given can only be approximate due to differences between batches of alumina.

Data compiled from Refs. [9], [21] and [44].

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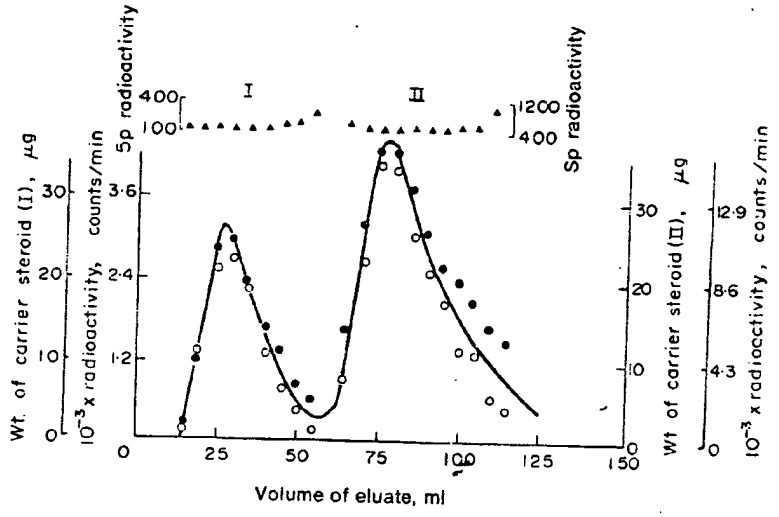

Fig. 16. Alumina-column chromatography, with light petroleum–benzene (1:1, v/v) as eluent, of an-α (I) and an-β (II) obtained after incubation of [4-^{14}C] progesterone with minced boar testis tissue. The weight of steroid (○) was determined by g.l.c. and radioactivity (●) by liquid scintillation counting; △, specific radioactivity (counts/min/μg). Redrawn from Ref. [6] by permission of *The Biochemical Journal*.

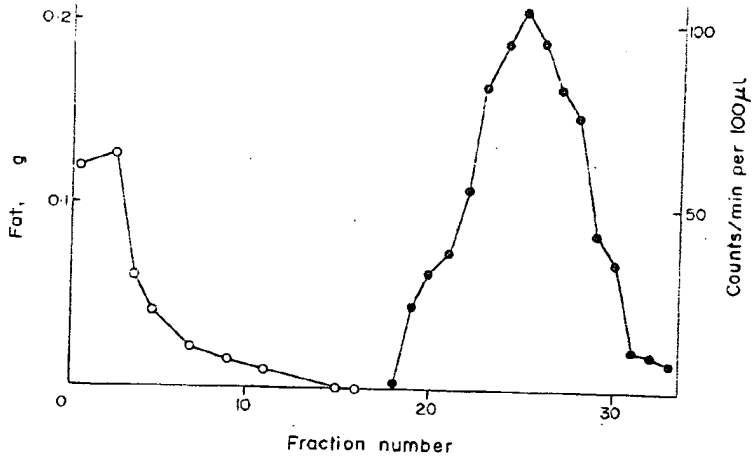

Fig. 17. Separation by silicic acid column chromatography of fat (○) from ^{3}H-labelled 5α-androstenone (●) extracted from boar parotid gland. The column was eluted using the following solvent mixtures (5 ml fractions):—cyclohexane (70 ml); light petroleum–cyclohexane (1:1, v/v) (15 ml); light petroleum (25 ml); light petroleum–ethyl acetate (1:1, v/v)) (10 ml) and ethyl acetate (15 ml). Redrawn from Ref. [4] by permission.

unpublished), but this pair of compounds can be resolved if a column of silicic acid impregnated with silver nitrate is used [8, 25]. An-β can be eluted using benzene–ethyl acetate (2:1, v/v) but the more unsaturated andien-β is avidly retained by the column and can only be eluted using benzene–ethyl acetate–ethanol (40:20:1, by vol). Such a system has been of value in showing that labelled andien-β was formed from labelled pregnenolone in incubations of human adrenal carcinoma tissue [25], human testis [71] and porcine testis [8] (Fig. 18). A positive pressure

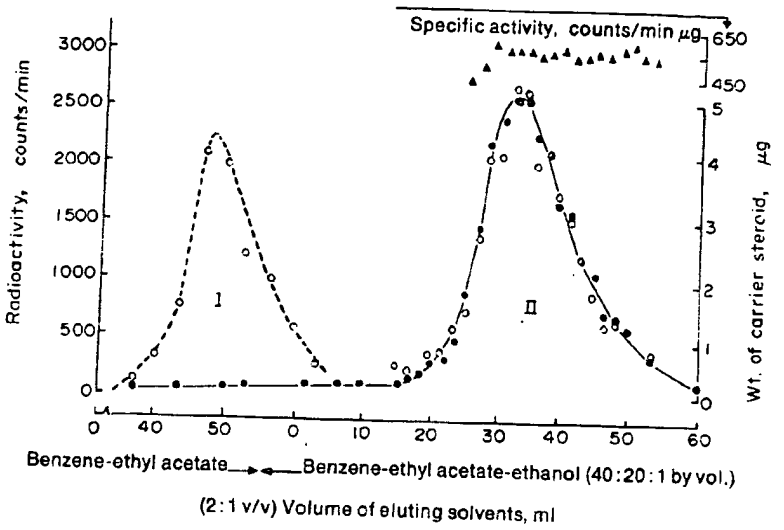

Fig. 18. Purification of radioactive andien-β formed by incubating [4-^{14}C] pregnenolone with adrenocortical carcinoma tissue. Radioactive andien-β was isolated by t.l.c., carrier an-β (I) and andien-β (II) added, and the mixture eluted from a column of Kieselgel H impregnated with $AgNO_3$ (for details see Refs. 8 and 25). The weight (○) of carrier steroids was determined by g.l.c. and radioactivity (●) by liquid scintillation counting. Reproduced from Ref. [25] by permission of Periodica, Copenhagen.

of N_2 (10–20 mm Hg) must be applied to such columns in order to obtain a flow rate of 1 ml/min.

(b) *Paper chromatography.* The 16-unsaturated C_{19} steroids are so non-polar that in the Bush A system [102] they run, as a group, near the solvent front [76, 77]. Gower [103] had previously encountered this problem and employed paper that had been fully acetylated or impregnated with kerosene, liquid paraffin or phenylcellosolve. However, it was only possible to separate an-α from ae-α, andien-β and an-β, these three compounds moving as a group. The situation was thus analogous to that described above for alumina column chromatography. Using silicic acid–impregnated paper, however, an-α and ae-α could be resolved from each other and both were separated from the still unresolved andien-β plus an-β, by ascending chromatography. Non-polar solvents or solvent mixtures, such as light petroleum–benzene (1:1, v/v), benzene, toluene and benzene–ether (99:1, v/v) were used [103]. The order of mobility for the 16-unsaturated steroids studied was oestratetraenol > an-α > ae-α > andien-β ≥ an-β. Using the descending technique for 18 h on silicic acid–impregnated paper, it was still not possible to resolve andien-β from an-β, although enhanced separations of the other compounds were achieved. The acetates, much less polar than the parent alcohols, were poorly separated in all the systems tried.

(c) *Thin layer chromatography.* When t.l.c. was attempted with 16-unsaturated steroids [103, 104] similar resolutions were achieved as with silicic acid–impregnated paper (Table 16). It was possible to effect reasonable separations of oestratetraenol, an-α and ae-α from andien-β and an-β, if the plate was developed in the same solvent system, e.g. benzene–ether (9:1, v/v), two or three times, allowing the plate to dry between runs. Andien-β and an-β were, however, only partially separated. 5α-Androstenone and androstadienone can also be readily

Table 16. R_F values (X100) of some 16-unsaturated steroids on Kieselgel G

	Solvents†																			Colours with detecting reagents‡						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	5	18	19	PMA*	PTA*	RA*	Allen*	Uranyl nitrate*	Anisaldehyde-H_2SO_4*	I_2 reagent*
An-α	8	29	21	34	24	33	66	40	75	61	74	52	68	59	29	51	36	28	48	—	bg	br	m	gr	gr	vb	yb
Ae-α	5	22	19	26	20	27	58	33	62	55	65	45	66	56	24	48	30	23	42	—	g	br	m	gr	bgr	vb	yb
Andien-β	4	18	16	23	17	23	49	28	49	47	59	40	66	53	21	45	27	19	38	—	p	ro	b	m	p	vb	yb
An-β	4	19	15	20	15	20	48	27	46	45	58	38	66	51	20	41	25	18	36	—	b	br	m	gr	p	vb	yb
Oestratetraenol	15	38	37	48	30	51	72	51	93	77	88	80	96§	—	—	—	—	—	—	21§	rp	o	r	bk	r	vb	yb

*PMA, phosphomolybdic acid; PTA, phosphotungstic acid; RA, resorcyaldehyde reagent; I_2 reagent, I_2 in light petroleum[103]; anisaldehyde reagent[104]. Allen reagent[107]; Uranyl nitrate[103].

†Solvents: 1, toluene; 2, methylene chloride; 3, toluene-ethyl acetate (19:1 v/v); 4, toluene:ethylacetate (9:1 v/v); 5, benzene-ethanol (98:2 v/v); 6, benzene:ether (9:1 v/v); 7, benzene-acetone (85:15 v/v); 8, benzene-methyl ethyl ketone (9:1 v/v); 9, methylene chloride-ethyl acetate (9:1 v/v); 10, ethylene chloride-ethyl acetate (90:12 v/v); 11, di-isopropyl ether-acetic acid (96:4 v/v); 12, di-isopropyl ether-formic acid (99:1 v/v); 13, ethyl acetate-cyclohexane-ethanol (45:45:10, by vol.); 14, ethyl acetate-cyclohexane (1:1 v/v); 15, *n*-hexane-ethyl acetate (75:25 v/v); 16, benzene-ethyl acetate (1:1 v/v); 17, benzene-ethanol (95:5 v/v); 18, chloroform-ethanol (98:2 v/v); 19, benzene.

‡Colours: b = blue; bg = bluish green, bgr = bluish grey; br = brown; g = green; gr = grey; m = mauve; o = orange, r = red; or = orange red; ro = rose; rp = reddish purple; p = purple; pk = pink; vb = violet blue; yb = yellow brown. Data from [36, 103, 104] by permission of the publishers (Elsevier Publishing Co; Academic Press Inc.).

§Detected by Kober reaction[36].

separated by this technique (Fig. 19). Using Kieselgel G layers impregnated with silver nitrate, it is possible to resolve andien-β from an-β, the former being much more retarded[104] (Table 17). In order to separate a range of 16-unsaturated steroids possibly formed in incubations of labelled substrates with testis or adrenocortical tissues, Gower and his colleagues[8,25,31,71] have utilised t.l.c. first on Kieselgel G using the systems benzene-ether (9:1, v/v) or benzene:ether (4:1, v/v) run twice, followed by t.l.c. on Kieselgel G impregnated with $AgNO_3$ in the system benzene:ethyl acetate (1:2, v/v).

However, although the relatively non-polar solvent systems separated the 16-unsaturated steroids, more polar compounds such as pregnenolone, progesterone, 17α-hydroxypregnenolone and testosterone were only partially separated, and had to be eluted 'en bloc' and re-run in systems such as benzene–acetone (4:1, v/v)[31]. In incubations of radioactive pregnenolone or progesterone all of these compounds were likely to be encountered. Thus, in order to separate, on one plate, the 16-unsaturated steroids from C_{21} and other C_{19} steroids, two-dimensional t.l.c. has recently been exploited[105]. Figure 20 shows the separation of some sixteen compounds and it is of particular interest that one curve can be drawn through the positions of all 4-en-3-oxosteroids; a second curve relates the positions of 5-ene-3β-hydroxysteroids. Similar separations have been achieved using extracts of testes from human testicular feminization patients[106] and from rats[105], after incubation with radioactive pregnenolone or progesterone.

(d) *Elution of 16-unsaturated steroids from thin-layer plates.* Using solvents such as chloroform, methylene dichloride, ethyl acetate or ether (4 × 2 ml), it is possible to recover approximately 80% of these compounds from Kieselgel G plates. However, if $AgNO_3$-impregnated Kieselgel G is used, the steroids, particularly the alcohols, are more strongly adsorbed and recoveries of as little as 20% for andien-β have been recorded in the author's laboratory. This difficulty was

Table 17. R_F values (×100) of some 16-unsaturated steroids on silver nitrate-impregnated Kieselgel G

	Solvents							
	1	2	3	4	5	6	7	8
An-α	10	37	6	30	21	15	3	22
Ae-α	6	34	6	15	8	7	3	10
Andien-β	9	32	4	25	14	12	3	17
An-β	14	37	11	31	23	18	4	28
Androstadienone	—	—	—	—	—	—	—	30
5-Androsten-3β-ol	51	67	26	57	50	45	24	63
Cholesterol	—	—	—	—	—	—	—	55
Pregnenolone	—	—	—	—	—	—	—	47
Progesterone	—	—	—	—	—	—	—	58
Oestratetraenol	—	—	—	—	—	—	—	45

Solvents: 1, chloroform-ethanol (95:5 v/v); 2, ethyl acetate-cyclohexane-ethanol (45:45:10 by vol.); 3, ethyl acetate-*n*-hexane (25:75 v/v); 4, ethyl acetate-*n*-hexane (75:25 v/v); 5, cyclohexane-ethyl acetate (1:1 v/v); 6, benzene-ethyl acetate (1:1 v/v); 7, benzene-ethanol (95:5 v/v); 8, benzene-ethyl acetate (1:2 v/v). Data from [104] for solvent systems 1–7 and from [8] for system 8 (by permission of the publishers, Academic Press Inc. and The Biochemical Journal).

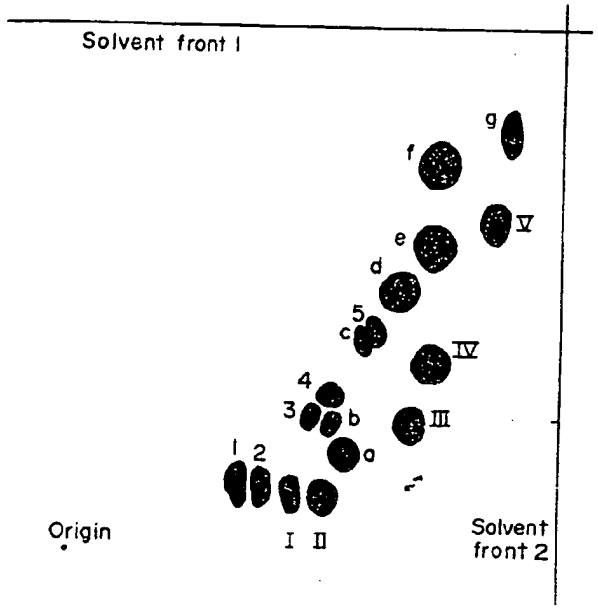

Fig. 20. Two-dimensional t.l.c. of a mixture of steroids using benzene-ether (9:1, v/v), run twice, as the first system and benzene-methanol (9:1, v/v) as the second. 4-en-3-Oxosteroids were I, testosterone; II, 17α-hydroxyprogesterone; III, androstenedione; IV, progesterone; V, androstadienone. 5-ene-3β-Hydroxysteroids were; −1, 17α-hydroxypregnenolone; 2, 5-androstenediol; 3, dehydroepiandrosterone; 4, pregnenolone; 5, andien-β. Other steroids: a, androsterone; b, aetiocholanolone; c, an-β; d, ae-α; e, an-α; f, oestratetraenol and g, 5α-androstenone.

overcome by mixing the appropriate zone of adsorbent material in a test-tube with distilled water (1·5 ml) and 2M-NaOH (0·5 ml) and extracting the steroid with ether (4 × 2 ml). Acceptable recoveries were then obtained[8].

An attempt was made[78] to use t.l.c. on $AgNO_3$–impregnated Kieselgel G to separate synthetic [7α-^{3}H] androstadienone from a reaction mixture containing impurities, possibly ring D isomers of androstadienone (see p. 78). Although an excellent separation of androstadienone was achieved, it seemed likely that the use of NaOH to extract the steroid from the plate caused decomposition (Gower, unpublished observations). As mentioned earlier (p. 78) no decomposition of [^{3}H]- or [^{14}C]-labelled androstadienone occurred on columns of $AgNO_3$-impregnated silicic acid, possibly because the columns were run in an atmosphere of nitrogen.

(e) *Detection of 16-unsaturated steroids on thin-layer plates.* The reagents that have been used to detect 16-unsaturated steroids on thin-layer plates, together with the colours obtained, are given in Table 16. Of the reagents that do not result in destruction of the steroids, iodine in light petroleum spray is undoubtedly the most useful, since it gives yellow-brown colours with oestratetraenol, 5α-androstenone, androstadienone, an-α, ae-α, andien-β and an-β at room temperature[103]. Moreover, the spots disappear after the plate has been allowed to stand at room temperature for approximately two hr. No quenching has been observed if such radioactive spots are eluted and subsequently counted in a liquid scintillation spectrometer. If however, iodine vapour is utilised, colour production is not reversible[103]. U.V. light (254 nm) is also useful in that androstadienone and oestratetraenol can be detected.

16-Unsaturated steroids can be detected on $AgNO_3$-impregnated layers if heated with Allen reagent[107] but radioactive spots (located by radioautography) must be eluted before heating at 110°C[8].

(f) *Gas–liquid chromatography.* The non-polar nature of the 16-unsaturated C_{19} steroids permits their elution from a variety of stationary phases in relatively short times at temperatures in the range 190–210°C. The behaviour of these compounds on g.l.c. columns was first studied by Baker and Gower[108] using a high proportion (10%) of silicone gum as stationary phase. The sequence of elution for the free steroids was ae-α < an-α ≤ andien-β ≤ an-β < oestratetraenol (Table 18). It was not possible to resolve an-α, andien-β and an-β from each other as free steroids. The trimethylsilyl (TMS) ethers, however, were found to be more volatile than the parent alcohols and consequently could be eluted at temperatures some twenty degrees lower. Later studies[29] involved the use of high concentrations (20%) of a more selective stationary phase (QF_1). It was then possible to separate the TMS ethers of andien-β and an-β and to show the absence of the latter compound in human urine. During recent years a variety of stationary phases (summarized in Table 18) have been used to separate and estimate not only the alcohols mentioned above but also the ketones, androstadienone and 5α-androstenone. These compounds are retained longer than the closely related alcohols andien-β and an-α if QF_1 and XE-60 are used. However, if other phases, such as SE 30, are used, the retention times of the ketones are similar to those of the 16-unsaturated alcohols. This large difference in relative retention time on different phases has been useful in suggesting the presence of an oxo-grouping in unknown compounds, in particular, androstadienone[5].

The chloromethyldimethylsilyl (CMDS) and bromomethyldimethylsilyl (BMDS) ethers of the 16-unsaturated C_{19} steroids have been studied recently [25, 109] and, using these derivatives, a separation of an-α and ae-α was achieved on CHDMS*/polysiloxane (JXR) (0·6%/0·75%). The derivatives of andien-β and an-β were, however, not separated on this hybrid phase but only on XE-60 or QF_1 (Table 18 and Fig. 21). These CMDS ethers were especially useful in that they were retained longer on the columns than were the corresponding free alcohols and consequently facilitated the quantitation of an-α from human urine, the impurities being eluted well before the an-α CMDS ether (Ref. [109], Fig. 22). Both the CMDS and the BMDS ethers are less volatile and more stable than the TMS ethers and for this reason, a method for the analysis of this steroid series [23] utilises g.l.c. of the CMDS ethers. The BMDS ethers are more polar than, and are retained longer than, the corresponding CMDS ethers[25] (Table 18) as anticipated by analogy with the C_{19} 17-oxo steroid series[110] and may well be of use in the electron capture determination of nanogram quantities of 16-unsaturated C_{19} steroids.

The pattern of elution of the halogenosilyl ethers on CHDMS/JXR is an-α < ae-α < andien-β = an-β, and on QF_1 or XE-60 is an-α ≤ ae-α < andien-β < an-β.

10. ESTIMATION OF 16-UNSATURATED C_{19} STEROIDS

(a) *Colorimetric methods.* For the estimation of an-α in urine Brooksbank and Haslewood[21] used a modification of the colour reaction described by Miescher [111]. Hydrolysed urinary glucuronoside fractions were purified on alumina and

*Cyclohexanedimethanol Succinate.

Table 18. Relative retention times of 16-unsaturated steroids and their derivatives on various stationary phases

	(MS 2211) Silicone gum						QF_1									
	15%		10%				5%		5%		5%		20%			
	Free steroids 185°C	Free steroids 206°C	Free steroids 185°C	TMS ethers 185°C	Acetates 185°C		TMS ethers 170°C	CMDS ethers 195°C	Free steroids 196°C	CMDS ethers 196°C	CMDS ethers 190°C	CMDS ethers 200°C	Free steroids 205°C	TMS ethers 205°C	Acetates 205°C	
An-α	5·37	5·04	2·77	3·05	3·72		0·24	0·59	0·325	0·610	0·606	0·750	3·37	3·05	3·72	
Ae-α	4·86	4·47	2·62	2·97	3·76		0·24	0·63	—	—	0·610	0·786	3·45	2·97	3·76	
Andien-β	5·25	4·96	2·83	3·53	4·18		0·25	0·73	—	—	0·730	0·910	3·40	3·53	4·18	
An-β	5·43	5·11	2·90	3·60	4·21		0·25	0·78	0·410	0·815	0·715	1·16	3·76	3·60	4·21	
Oestratetraenol	6·81	5·96	3·63	4·18	5·02		0·26	0·95	—	—	—	—	4·39	4·18	5·02	
5α-Androstenone	—	—	—	—	—		—	—	—	—	—	—	—	—	—	
Androstadienone	—	—	—	—	—		—	—	1·24	—	—	—	—	—	—	
Retention time of standards (min.) ≅ 1·00	MP 4·0	MP	MP 9·35	MP 9·35	MP 9·35		C 52·0	C 20·6	C 18·3	C 18·3	C 35·9	C 33·0	MP 14·1	MP 14·1	MP 14·1	
Reference	[108]	[108]	[29]	[29]	[29]		[108]	[108]	[5, 20]	[5, 20]	[25]	[25]	[29]	[29]	[29]	
Flow rate (ml/min)	40	35	26	26	26		50	50	50	50	50	50	18	18	18	
Column dimensions (length × i.d. in cm)	←——— $122 \times 0{\cdot}35$ ———→						←— $213 \times 0{\cdot}4$ —→		←— $150 \times 0{\cdot}4$ —→		←— $150 \times 0{\cdot}4$ —→		←——— $168 \times 0{\cdot}3$ ———→			

Table 18 (*cont.*)

	NPGS 2%			CHDMS 1%		XE-60 1%	XE-60 2%			XE-60 2·5%			
	Free steroids 230°C	TMS ethers 230°C	Acetates 230°C	TMS ethers 170°C	CMDS ethers 195°C	Free steroids 180°C	Free steroids 180°C	TMS ethers 170°C	CMDS ethers 190°C	Free steroids 196°C	Methyl oximes 196°C	CMDS ethers 196°C	BMDS ethers 196°C
An-α	6·17	2·21	5·35	0·15	0·53	—	4·91	1·82	0·62	0·34	—	0·610	0·750
Ae-α	5·80	2·61	5·43	0·15	0·70	—	5·07	2·27	0·715	—	—	0·630	—
Andien-β	6·50	3·11	6·18	0·16	0·87	—	5·60	2·66	0·85	—	—	0·725	—
An-β	6·43	3·10	6·42	0·16	0·87	—	5·57	2·67	0·88	0·39	—	0·792	—
Oestratetraenol	—	4·94	—	0·38	1·72	—	—	—	—	—	—	—	—
5α-Androstenone	—	—	—	—	—	—	—	—	—	0·77	0·344	—	—
Androstadienone	—	—	—	—	—	2·12	—	—	—	1·18	—	—	—
Retention time of standards (min)	MP	MP	MP	C	C	A	A	A	C	C	C	C	C
≡ 1·00	5·3	5·3	5·3	51·6	18·1	4·6	1·95	2·66	14·1	20·75	20·75	20·75	20·75
Reference	[29]	[29]	[29]	[109]	[109]	[32]	[23]	[23]	[23]	[5,20]	[5,20]	[5,20]	[5,20]
Flow rate (ml/min)	17	17	17	50	50	50	50	50	50	50	50	50	50
Column dimensions (length × i.d. in cm.)	← 173 × 0·3 →			← 150 × 0·35 →		150 × 0·4	← 150 × 0·4 →			← 150 × 0·4 →			

Table 18 (*cont.*)

	CHDMS/JXR (0·6%/0·75%)												
	TMS ethers 170°C	TMS ethers 170°C	Free steroids 180°C	Free steroids 190°C	Methyl oximes 190°C	CMDS ethers 190°C	CMDS ethers 195°C	Free steroids 196°C	Methyl oximes 196°C	CMDS ethers 196°C	BMDS ethers 196°C	CMDS ethers 200°C	BMDS ethers 200°C
An-α	0·09	2·02	3·68	—	—	0·415	0·41	0·228	—	0·402	0·532	0·412	0·580
Ae-α	0·09	2·36	3·51	—	—	0·488	0·51	0·210	—	0·437	—	0·475	0·677
Andien-β	0·10	2·84	4·23	—	—	0·580	0·62	0·25	—	0·545	0·748	0·586	0·840
An-β	0·10	2·90	3·95	—	—	0·580	0·63	0·243	—	0·545	0·748	0·591	0·840
Oestratetraenol	0·27	—	—	—	—	—	0·96	—	—	—	—	—	—
5α-Androstenone	—	—	3·99	—	—	—	—	0·254	0·250	—	—	—	—
Androstadienone	—	—	5·97	1·79	5·03† 5·52	—	—	0·357	—	—	—	—	—
Retention time of standards (min)	C	A	A	AO	A	C	C	C	C	C	C	C	C
= 1·00	94·5	3·94	2·66	9·3	2·4	32·5	34·3	21·5	21·5	21·5	21·5	17·6	29·8
Reference	[108]	[29]	[23]	[32]	[32]	[25]	[108]	[5]	[5]	[5]	[5]	[23]	[25]
Flow rate (ml/min)	←						50						→
Column dimensions (length × i.d. in cm.)	←						150 × 0·4						→

Table 18 (*cont.*)

	JXR/Epon (2%/0·2%)					
	Free steroids 190°C	Methyl oximes 190°C	Free steroids 200°C	TMS ethers 180°C	CMDS ethers 200°C	
An-α			2·17	2·05	4·67	
Ae-α			2·01	2·22	5·07	
Andien-β			2·27	2·69	6·10	A, 5α-androstane;
An-β			2·29	2·68	6·29	AO, 5α-androstan-17-one;
Oestratetraenol			—	—	—	C, 5α-cholestane;
5α-Androstenone			2·60	—	—	MP, methyl palmitate;
Androstadienone	1·44	3·37	3·68	—	—	CHDMS, cyclohexanedimethanol succinate;
Retention time of standards (min) ≡ 1·00	AO	A	A	A	A	JXR, polysiloxane gum; XE-60, cyanoalkyl silicone;
	8·8	3·3	3·61	6·56	3·61	QF_1, fluoralkyl silicone;
Reference	[32]	[32]	[23]	[23]	[23]	NPGS, neopentylglycol sebacate;
Flow rate (ml/min)	50	50	50	50	50	Epon, epoxy resin
Column dimensions (length × i.d. in cm.)	← 150 × 0·4 →					

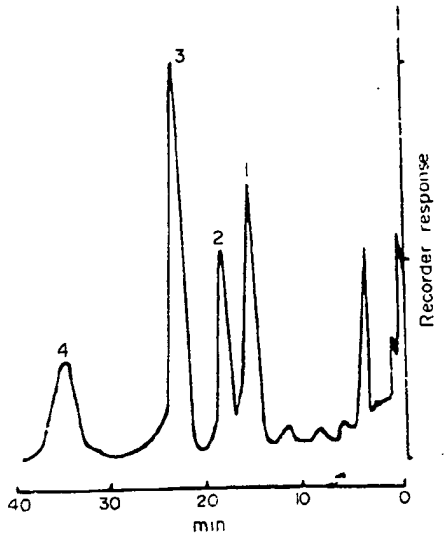

Fig. 21. G.l.c. of CMDS ethers of an-α (peak 1), ae-α (peak 2), andien-β plus an-β (peak 3) and oestratetraenol (peak 4).

Column: CHDMS/JXR (0·6%/0·75%) on Chromosorb W (100–200 mesh) at 195°C and carrier gas flow 50 ml/min. Reproduced from Ref. [109] by permission of Elsevier Publishing Co.

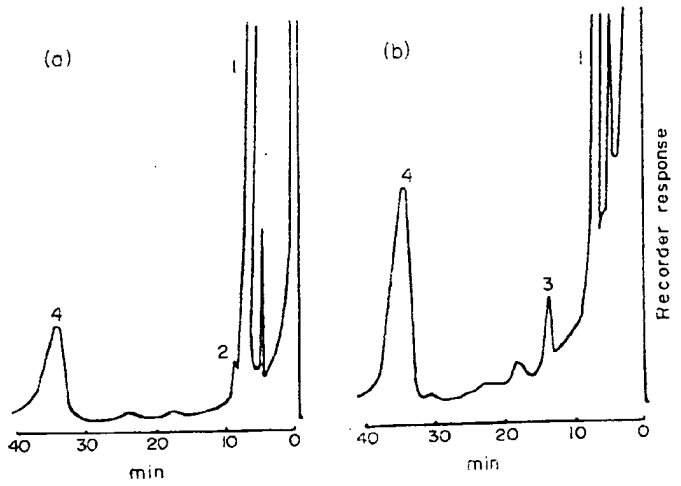

Fig. 22. G.l.c. of the an-α fraction, obtained from alumina column chromatography, of a normal female urine (a) before and (b) after chlorosilanisation (sensitivity twice that in (a)). Peak 1, unknown; peak 2, an-α; peak 3, CMDS ether of peak 2; peak 4, cholestane (internal standard). Column conditions as in Fig. 21 except that temperature was 197°C. Reproduced from Ref. [109] by permission of Elsevier Publishing Co.

an-α eluted with benzene–light petroleum (1:1, v/v) (see p. 78). The 'an-α' fraction was heated with resorcylaldehyde and concentrated sulphuric acid in glacial acetic acid, when a purple colour was produced that showed an extinction maximum at 580–585 nm, with an inflexion at 540 nm. A variety of C_{21} and C_{19} steroids were also tested with the same resorcylaldehyde reagent; only 16-unsaturated or 17α-hydroxy C_{19} steroids gave a purple, or in some cases (e.g. andien-β), a blue colour. Other steroids, apart from oestratetraenol (see Fig. 23),

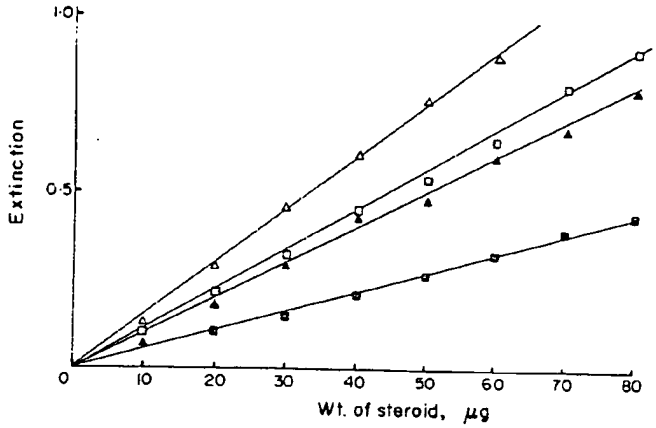

Fig. 23. Calibration curves for some 16-unsaturated steroids using the resorcylaldehyde colour reaction of Brooksbank and Haslewood[21]. Extinctions were measured at the following wave lengths: — ae-α (▲), 585 nm; andien-β (□), 575 nm; an-β (△) 585 nm; oestratetraenol (■), 625 nm. For the sake of clarity the curve of an-α (21) which closely follows that for ae-α has been omitted from the Figure. Data from Gower (unpublished).

gave essentially no colour. Cholesterol in large quantities (100 μg) gave a pink colour but was readily separated from an-α by alumina chromatography (Table 15) so that no interference from it was experienced when a urinary analysis for an-α was performed. However, it was found that the an-α fraction from urine extracts purified on alumina sometimes gave a pinker colour at lower wavelengths (530 nm) than did pure an-α. The observed extinction at 575 nm was therefore corrected for the extinction of interfering chromogens, but for very low titre urines containing 100 μg or less an-α per 24 h, the values obtained were in error even after using the correction factor. The same applies to the quantitative analysis of urinary ae-α and andien-β which occur to a much smaller extent than an-α. Although linear relationships are obtained between the amount of ae-α and andien-β and extinctions at 575 or 585 nm (Gower, unpublished, Fig. 23), the combined values for ae-α plus andien-β obtained by the colorimetric method were some five times greater than the individual values obtained by g.l.c.[29].

Recently, an attempt was made to utilise the same colour reaction for the estimation of 5α-androstenone but the sensitivity was insufficient for measurement of the compound in the peripheral plasma of pigs[4].

(b) *Gas–liquid chromatographic methods*. The behaviour on g.l.c. columns of 16-unsaturated C_{19} steroids has been described earlier (p. 86). The responses of some of these compounds and their derivatives to flame ionization detectors have been found to be linear. Calibration curves for some alcohols, silyl ethers and ketones are shown in Fig. 24. A number of compounds have been employed as internal standards in the g.l.c. estimation of 16-unsaturated steroids e.g. methyl palmitate, 5α-androstane, cholestane, 5α-androstan-17-one, 5α-androstan-3β-ol and 5α-androstan-17β-ol. The latter alcohols are particularly useful if TMS ethers or halogenosilyl ethers have to be prepared during the method since their use can correct for the losses that may be incurred during the silylation. An-α, ae-α and andien-β have thus been estimated in both normal and abnormal human urine [23–25,31,71] and an-β has been estimated in boar urine[5]. G.l.c. has also been helpful in the estimation of an-α, ae-α, andien-β and an-β as well as androstadienone and 5α-androstenone, added as carriers to *in vitro* incubations[6,8,9,12].

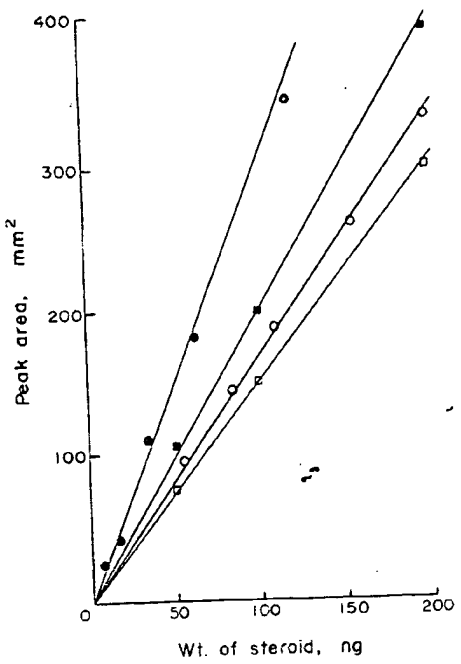

Fig. 24. Responses of some 16-unsaturated steroids and derivatives to flame ionization detection:– an-α (○), an-β (□), androstadienone (■), ae-α TMS ether (●). The gas chromatograph used was the dual flame ionization, model 24 (Pye-Unicam Ltd. Cambridge) with attenuation $\times 50$ (full scale deflection 5×10^{-11} A). Data from Gower (unpublished).

G.l.c. methods have been utilised for the measurement of some 16-unsaturated steroids in pig peripheral plasma, boar testis and salivary glands[3,4]. The concentrations of an-α, an-β and 5α-androstenone have been determined in boar spermatic vein plasma[5] and the concentration of androstadienone in human peripheral plasma has likewise been measured by a g.l.c. method[32].

(c) *Protein-binding methods*. Attempts to estimate 5α-androstenone in pig peripheral plasma by protein-binding have so far been unsuccessful[4]. It seems likely, however, that an-α may be able to displace testosterone from testosterone binding globulin in bovine plasma[4].

11. PRACTICAL IMPLICATIONS OF THE ODOUR OF 16-UNSATURATED C_{19} STEROIDS

Prelog and Ruzicka[82] commented upon the musk-like odour of an-α and an-β and described that of an-α as being more intense than that of an-β. Subsequent studies[84] showed that the corresponding 5β-isomers were odourless and that the ketones, androstadienone and 5α-androstenone, possessed pronounced urine-like or perspiration-like smells. These authors also drew attention to the superficial structural similarities between 5α-androstenone and civetone (Fig. 1). The characteristic odours of this group of compounds have been useful in detecting the presence of very small quantities in tissue, plasma or urine extracts. For example, 5α-androstenone was detected in boar fat extracts as it emerged from a g.l.c. column[13]; Brooksbank and Haslewood[2] were able to smell the musk-like odour of a compound (later identified as an-α) in hydrolysed urine

extracts; and other workers[5] described how the smell of 5α-androstenone could be detected on the hot syringe needle after a boar spermatic vein plasma extract had been injected on to a g.l.c. column. However, the characteristic musky smell is not restricted to 16-unsaturated alcohols; the 3-hydroxy-5α-androstanes also possess a musk-like odour[84], although the 5β-epimers are odourless and, out of 33 steroids investigated by Beets[112], the musk odour was detected in thirteen derivatives of androstane. The olfactory properties of the 16-unsaturated steroids has been referred to in a number of papers[113–116] and has been reviewed recently[56]. The olfactory response of men, women and children to the musky odour of the macrocyclic ketone, exaltolide, has been intensively studied[117, 118]. The ability of women to smell this compound increases during the follicular phase of the menstrual cycle, reaches a maximum at ovulation but declines during the progestational phase. Le Magnen[117] has also shown that children, men and post-menopausal or oophorectomized women can only smell exaltolide faintly. Administration of oestrogens, however, causes a recovery in the olfactory acuity. In a more recent study of 73 female student nurses[118] two peaks occurred in olfactory acuity, one just preceding ovulation (17 days before the menses) and the other during the luteal phase (8 days before the menses). Such experiments[117, 118] suggest that there may be a link between oestrogens and the ability to smell musky odours.

In 1948, an interesting case of anosmia to an-α was described[119] and a more detailed study of 200 men and women[120] revealed that 29% of the men were anosmic to an-α, 38% described the smell as faint and 33% as strong. The corresponding percentages for women were 22, 36 and 42%. The results of a similar study using 5α-androstenone were more clear cut[121]. In this investigation records were made of the olfactory response of 50 men and 50 women to 800 ng. of the pure compound that had been applied (in ethereal solution) to 5 cm^2 of a watch-glass. It was found that 44·3% of the men were unable to detect the odour in contrast to only 7·6% of the women. Most of the female subjects rated the smell as extremely unpleasant. A high proportion of women also find the smell of roasting boar meat unpleasant[122] since the odour of the 5α-androstenone in the fat (see p. 47) is especially noticeable when the meat is hot. This has important practical implications, as discussed recently[121], since women, rather than men, are most often involved in the preparation and cooking of pork or bacon and will decide whether it is acceptable. However, when the meat has reached the table and cooled somewhat, the intensity of the odour may have decreased and the meat may then be more palatable. In this connection, it is of interest that the German cartoonist Wilhelm Busch (1832–1908) has alluded to the great sensitivity of women to the smell of pigs. In a cartoon entitled "Ebergeruch" (pig smell), a herd of pigs is shown being to market. The woman near them holds up her hands in horror at the smell (depicted as a spirit), while the man walks on, apparently unconcerned.

The unpleasant smell and flavour of cooked meat taken from an uncastrated or partially castrated boar was described many years ago[123, 124]. One investigator [124] considered that the submaxillary glands possessed the unpleasant taste and recommended their removal; another described the parotid glands as possessing the bad odour even when the fat and meat were considered as negative. By smelling the meat and melted fat separately at different times after castration, it was evident that the smell persisted for two months. Cryptorchid animals have

also been shown to possess the smell[124, 125]. As discussed earlier (p. 63) the situation regarding boar salivary glands and 16-unsaturated steroids has still not been clarified. That the so-called 'sex-odour' was sex-dependent was shown by Williams and his collaborators[126] who found that 64% of males possessed the 'sex-odour' substances but only 1–5% of females and castrates. Experiments[62] performed in 1959 led to the suggestion that boar 'sex-odour' emanated from the preputial gland, since its surgical removal reduced the odour. However, the gland possesses no 16-unsaturated steroid biosynthetic activity[20] (see p. 63) nor does it contain more than traces of these steroids[65]. Patterson therefore believes that the smell of the gland is due to the presence of phenols, especially p-cresol[64] and fatty acids[65] (see p. 63).

Although the sex-odour-producing substances were known to be water-insoluble and ether-soluble and to be present in the unsaponifiable fraction of boar fatty tissue extracts[127, 128], attempts to characterize the odorous principle were unsuccessful[129]. It was not until 1968 that 5α-androstenone was isolated and characterized as the 'boar taint' ketone[13] (see p. 47). The high vacuum distillation apparatus used in these experiments, however, did not give quantitative yields of the ketone. For this reason, a pot-still was used in later experiments [130] in which the ketone and other volatile compounds, removed from the molten fat under high vacuum, were trapped on a glass surface (cooled by liquid nitrogen at -196°C). The distance between the heated fat and the cooled surface was made as short as possible to increase the efficiency ($92 \pm 5\%$) of the process. A clean-up procedure with aqueous alkali, followed by t.l.c., was found to be necessary before the non-saponifiable material could be subjected to g.l.c. for final analysis (cf. the method used for human axillary sweat[34]).

Since boar 'sex-odour' is thought to originate principally in the testis, castration is normally performed during the first few weeks of life. Recent results[131] show that partial castration is as effective as complete castration in eliminating sex-taint in pork meat. In one group of animals, only testicular parenchymal tissue was removed; from another group, the testes were removed but the epididymis left intact. In both groups, little or no taint could be detected. However, although the principal site of formation of the odorous 16-unsaturated steroids is removed by such procedures, the formation of testicular androgens is also eliminated and it has long been known[132] that this results in a smaller percentage of lean meat, more back-fat, lower live-weight and a reduction in the quality of the meat. Another point worth consideration is that castration will result in lack of an-α (see p. 46), a compound that has a definite myogenic effect (Table 19). For these reasons, some attempts have been made to suppress the odour of the live boar without resorting to castration. Administration of stilboestrol and of 17α-methyl testosterone[133–135] were indeed shown to reduce the 'sex odour' in boars and a more recent study[136] confirmed that implantation in boars of 96 mg diethyl-stilboestrol at a live weight of 70·3 kg reduced boar odour even though plasma androgen levels were unaffected. Chlormadinone acetate (20 or 30 mg daily for periods of 33–70 days) also significantly decreased the taint of boar meat (tested by smell and taste) but the fat content of the meat doubled[137]. Such methods, however, are expensive and tedious to perform during the life of the pig up to the time of slaughter, and, as mentioned above, castration of most males is normally performed at an early age. Whether stilboestrol and 17α-methyl testosterone have an effect on the biosynthesis of 16-unsaturated steroids has not yet been estab-

lished but such an investigation is at present being undertaken in the author's laboratory. The problems connected with the taint in pork have been the subject of a number of recent reviews and papers[4, 138–140]. In particular the work of Elsley and Livingstone[140] has shown that the taint of boar fat is rarely detected in immature pigs of less than 43 kg body weight (< 100 days old) but thereafter it becomes increasingly common and there is a correlation between taint and age between 135 and 365 days, i.e. in animals weighing more than 92 kg (cf. Table 1).

Possible physiological role of 16-unsaturated steroids in pigs

In 1961 Signoret and du Mesnil du Buisson[141] showed that boar odour was necessary to elicit the characteristic mating stance of the sow *in oestrus* when subjected to the usual back-pressure test[142]. Over 81% of oestrus females so tested responded when kept in a pen previously occupied by a male. Further experiments[143] showed that the stimulus was an olfactory one, since surgical removal of the olfactory bulbs of the female abolished the response and, moreover, interfered with maintenance of the genital tract and inhibited the release of FSH from the anterior pituitary.

Since the period during which the female is *in oestrus* is relatively short, it is obviously of great importance for pig breeders to ascertain with the minimum delay if the sow is ready for artificial insemination without having to expose her to boar odour. Both boar urine and seminal fluid[143, 144] have been rubbed on to the snouts of sows and have been shown to induce the mating stance even in a proportion of recalcitrant animals (i.e. those that did not respond immediately to the normal back-pressure test of Altmann[142]). Such procedures, although effective, are not hygienic.

The penetrating smell of the 16-unsaturated steroids and their possible relationship to boar 'sex-odour' led Sink[145] to propose that these steroids might be acting as sex-attractants. Shortly afterwards[16] an-α was discovered in boar saliva and it was suggested that, as the boar becomes sexually excited and salivates profusely, the smell of the alcohol on the breath of the males reaches the female and elicits the characteristic immobilization response. The male is then able to mount and copulate. an-α and 5α-androstenone are the compounds that elicit the response in the female, was shown by Melrose, Patterson and Reed [146, 158] who sprayed 5α-androstenone and an-α separately, in aerosol form, towards the snouts of recalcitrant females and found that approximately 50% then responded to the back-pressure test. Artificial insemination can thus be carried out more efficiently and expeditiously than would otherwise be the case.

It is of interest that the musk-smelling an-β occurs in boar urine[5] and this may explain the effect (mentioned above) of the smell of such urine on sows *in oestrus*. It is also possible that 16-unsaturated steroids may occur in boar seminal fluid, their smell eliciting the characteristic response in the female.

Recent work reviewed by Signoret[147] has revealed that the reproductive behaviour of pigs is extremely complex. The female *in oestrus* requires not only the odour of a boar to elicit the mating stance; the sound of its 'courting song' and the sight of it are also important. Even the frequency of the males' grunts is important, since a tape-recording of a "song" with the frequency of grunts reduced by 50% causes a much lower frequency of characteristic responses when played back to the female.

16-unsaturated steroids as androgens

Since an-α is structurally related to the androgens and is also excreted in human urine in milligram quantities, this compound was for many years considered as possessing androgenic properties. The physiological effects of an-α, an-β and androstadienone have now been studied by bio-assay [148] (Table 19) and summarized recently [32,56]. It is clear that, at least in rats, rabbits and mice, an-α, an-β and androstadienone, show little or no androgenic activity. Recently, 5α-androstenone was also shown to have no androgenic activity compared with testosterone in the chick comb test [4]. Androstadienone was, however, shown to be weakly oestrogenic and this is explained on the basis of its possible conversion to oestratetraenol (p. 73) and of the latter's oestrogenic activity (see below). The physiological role of this group of compounds in these species and in humans therefore remains enigmatic. However, it is of interest that the powerfully-smelling 5α-androstenone has been found in the axillary sweat of a man (Ref. [34], see p. 55) but whether this compound plays any role as a sex-attractant remains to be elucidated. In this connection it is of particular interest that in some folk dances performed in Mediterranean countries, the male dancers stimulate their female partners by waving in front of them handkerchiefs that have been held for some time under the armpits of the men [149]. The nature of the active principle so obtained on the handkerchiefs is unknown but it is tempting to speculate that a blend of 5α-androstenone and other secreted substances may play a role in this respect. The smell of the ketone itself would presumably not produce the required stimulation in the women since many find the smell unpleasant [121].

Oestrogenicity of oestratetraenol

The relationship of this compound to epioestriol has already been described

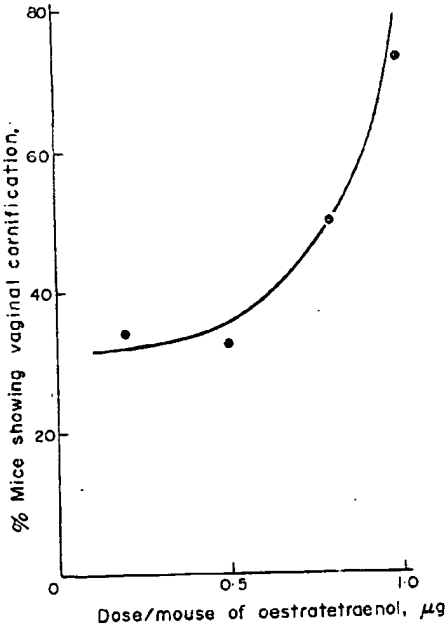

Fig. 25. Dose-response curve for oestratetraenol. Ovariectomized mice were given a single sub-cutaneous injection of oestratetraenol in sesame oil and vaginal cornification recorded.

Table 19. Physiological activity of 16-unsaturated steroids

	Androgenic	Anti-androgenic	myogenic***	Anti-myogenic	progestational	Uterotropic†	Anti-uterotropic†	oestrogenic	glycolytic, mineralocorticord, thymolytic, anti-inflammatory, anti-ACTH
Androstadienone	none*	none	none	none	none	none	trace (at 0·01 mg/3 days against 0·32 μg oestrone)	moderate†† (9 mg/3 days)	none
an-α	none*	very slight	100% as effective at 6 and 60 mg/7 days against 1–2·4 mg testosterone	moderate	none	none	none	none	none
an-β	none*	none	none	none	slight (at 8 mg/5 days against 0·32 μg oestrone)	none	none	none	none
5α-androstenone	none**						slight (at 9 and 10 mg/3 days against 0·32 μg oestrone)		

*wt. of accessory sex organs of castrated male rats; **chick comb test; ***wt. of levator ani of castrated male rats.
†wt. of uteri of immature female Swiss albino mice; ††ovarian wts. of immature New Zealand white rabbits.
Data from refs. 148 and 4.

(p. 73) and its oestrogenicity in rats has been studied briefly by a number of workers[96. 150]. More recent investigations[151] have shown that, in mice, oestratetraenol was much less oestrogenic than oestradiol-17β, the threshold dose being 0·2–0·5 μg per mouse. A dose of 1·0 μg caused 76% of mice injected to go into oestrus and caused a significant increase in uterine weight compared with a control group. The dose-response curve (Fig. 25) was similar to that for oestradiol, approximately 1·0 μg oestratetraenol per mouse being equivalent to 0·1 μg of oestradiol-17β.

12. SUMMARY AND CONCLUSIONS

The experiments described here, that have been performed by workers in the disciplines of chemistry, biochemistry, physiology and agriculture, have made it abundantly clear that the 16-unsaturated C_{19} steroids are of great physiological importance, at least in pigs. The question as to whether these compounds may act as pheromones in humans, through excretion in the sweat or urine, has been discussed and, for the present, remains open. Further problems still exist, such as the significance, or otherwise, of the increased amounts excreted in endocrine disorders such as virilizing adrenal carcinoma and especially the source of an-α. Undoubtedly, further research will help to solve the mystery that still pervades this fascinating group of compounds.

NOTES ADDED IN PROOF

p. 47. Recent work [152. 153] has now revealed that boar fat contains an-α in addition to 5α-androstenone.

p. 48. Analyses of parotid glands taken from Landrace boars (Ref. 154; D. B. Gower and Y. A. Saat, unpublished observations) have shown the presence of only very small quantities of 16-unsaturated C_{19} steroids. These results contrast markedly with those of Claus [4] (see Table 1). Boar parotid glands, however, contain a high proportion of fat and, if this is separated and analysed, it is found to contain a much higher concentration of 16-unsaturated C_{19} steroids than the parotid gland tissue itself (D. B. Gower and Y. A. Saat, unpublished observations). It is possible that the discrepancy between the available analytical results may be explicable in the light of these recent findings.

p. 64. More detailed analyses[155] of boar testes that had been infused with [4-^{14}C]pregnenolone have shown the presence in these tissues of the sulphates of an-α and an-β (labelled with ^{14}C) in addition to the unconjugated alcohols[101]. In keeping with analytical results (Table 1), the amount of ^{14}C-an-β sulphate found exceeded that of the 3α-compound.

pp. 61 and 72. The reduction of [7α-^{3}H]androstadienone and [5α-^{3}H]5α-androstenone has recently been studied in preparations of boar testis[156. 157] and salivary glands[154]. In boar testis homogenates, kinetic studies revealed that androstadienone was converted first to 5α-androstenone (a reduction requiring NADPH) and that this was subsequently metabolised to a mixture of an-β (the major product) and an-α. The formation of an-α from 5α-androstenone was shown to be NADPH-dependent whereas the formation of an-β was NADH-dependent. These results are in excellent agreement with studies *in vivo*[101] and clearly establish androstadienone and 5α-androstenone as intermediates in 16-unsaturated C_{19} steroid biosynthesis (Fig. 6). By incubating minces of boar submaxillary and parotid glands, mixtures of an-α and an-β were also formed

from both ^{3}H-androstadienone or ^{3}H-5α-androstenone [154]. Using submaxillary gland tissue, the reduction of 5α-androstenone to an-α was shown to be NADPH-dependent, although NADPH or NADH seemed to be equally as effective in promoting the reduction when parotid gland was used. In contrast to earlier results (Ref. 7, see p. 19) no 16-unsaturated C_{19} steroids were formed when [4-^{14}C]pregnenolone was incubated with boar submaxillary gland minces [154]. Testosterone and DHA were likewise ineffective precursors *in vitro* [154].

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Thank you

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THE MEASUREMENT OF PREGNANEDIOL SULPHATE IN AMNIOTIC FLUID

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Although the only conjugate of pregnanediol (5β pregnane-3α,20α-diol) in urine is the glucosiduronate (Crepy, Judas, Rulleau-Meslin & Jaylo, 1962) when a foetus is perfused with progesterone some pregnanediol sulphate is formed (Bird, Solomon, Wiqvist & Diczfalusy, 1965). There is thus a possibility that pregnanediol sulphate is largely a foetal product and that its measurement may give some indication of foetal well-being. The identification of pregnanediol sulphate in liquor amnii (Solomon & YoungLai, 1969) and the development of a method for the measurement of all the pregnanediol conjugates in amniotic fluid (Klopper, Wilson & Shearman, 1969) has given rise to the opportunity of examining this possibility. In order to do so it was necessary to find out whether the solvolysis technique of Burstein & Lieberman (1958) for ketosteroid sulphates could be adapted to the separation of pregnanediol sulphate and pregnanediol glucosiduronate. We report here the results of such an investigation and the reliability data of the method for the assay of pregnanediol sulphate.

In essence the method of Klopper *et al.* (1969) for the estimation of total pregnanediol in amniotic fluid consists of extraction of the steroid by Delsal's reagent, acid hydrolysis, chemical purification, column chromatography on alumina first as the free alcohol and then as a diacetate and final measurement by gas–liquid chromatography. The problem was, therefore, to design and test a step whereby pregnanediol sulphate could be quantitatively solvolysed and removed for further analysis before the remaining conjugated fraction was submitted to acid hydrolysis.

In the method which was finally evolved 5 ml. of fresh unfiltered amniotic fluid is added to 10 ml. Delsal's reagent (4 parts methylal + 1 part methanol). The mixture is left to stand for an hour, centrifuged, decanted and the solid residue extracted with a further 10 ml. Delsal's reagent. The combined solution is taken to dryness at 50° under reduced pressure on a rotary evaporator. The residue is then partitioned between 10 ml. water and 10 ml. of an equal mixture of hexane and toluene. The hexane–toluene extract is assayed for free pregnanediol by the method of Klopper *et al.* (1969) and the aqueous phase submitted to solvolysis. For this purpose 4 g. NaCl and 1 ml. N-sulphuric acid are added to the water which is extracted twice with 20 ml. ethyl acetate. Approximately 50,000 dpm of [1,2-^{3}H]pregnanediol is added to the ethyl acetate as an internal standard and the solution is warmed in a waterbath at 56° for an hour. The ethyl acetate is then washed with 10 ml. N-NaOH and twice with 10 ml. water. The combined washings are submitted to acid hydrolysis and further

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analysed as the glucosiduronate fraction according to the method of Klopper *et al.* (1969). A check of the radioactivity remaining in the ethyl acetate showed that no significant amount of free pregnanediol is removed by the water washing. The ethyl acetate is evaporated and the solvolysed pregnanediol sulphate assayed by the method of Klopper *et al.* (1969).

The accuracy of the method was tested by the recovery of 25·9 μg. pregnanediol sulphate added to 5 ml. liquor. The authentic pregnanediol-3 sulphate was kindly prepared by Dr A. Turner of the Chemistry Department in the University of Aberdeen. In six replicate experiments the radioactive internal standard gave an average recovery of 92·5%. Each individual sample was corrected by its radioactive internal standard. The average corrected recovery of non-radioactive pregnanediol sulphate was 82·2% with a range of 75·4 to 87·4%.

There are two aspects to be considered concerning the specificity of the technique. The gas chromatographic peak read in the method of Klopper *et al.* (1969) has been examined by mass spectrometry and found to consist of pregnanediol. This technique, therefore, is specific for the pregnanediol moiety and it remained to be shown that the sulphate fraction is not contaminated by pregnanediol coming from the free or glucosiduronate fraction. When free pregnanediol was added to the liquor there was no additional pregnanediol recovered in the sulphate fraction. Whether or not any pregnanediol coming from the glucosiduronate fraction appears in the sulphate fraction is difficult to prove as no authentic pregnanediol-3 glucosiduronate is available for testing. It has, however, been conclusively shown that pregnanediol in pregnancy urine is present as the 3α-yl-β-D-glucopyranosiduronate (Heard, Hoffmann & Mack, 1944). The yield from a pregnancy urine extract when put through the solvolysis procedure was, therefore, examined. For this purpose 200 ml. of late pregnancy urine was extracted by the method of Kellie & Wade (1956). The solvent was evaporated and the residue put through the solvolysis procedure. The ethyl acetate phase was found to contain traces of pregnanediol (1·27 μg.) possibly from solvolysis of conjugates. Acid hydrolysis and analysis of an aliquot of the aqueous phase showed that it contained a substantial amount of conjugated pregnanediol (1·04 mg.), presumably in the form of glucosiduronate. Aliquots of this glucosiduronate solution containing 26 μg. pregnanediol were added to 5 ml. of liquor and put through the method. On this occasion the sulphate fraction yielded 0·55 μg. pregnanediol above the blank level due to the liquor alone while the water washings of the ethyl acetate contained 20·1 μg. of conjugated pregnanediol. This experiment shows that the glucosiduronate fraction of pregnancy urine is not hydrolysed by the solvolysis procedure and does not appear in the sulphate fraction but can be substantially recovered by subsequent acid hydrolysis of the water washes. The overall findings indicate that this technique is adequate for the measurement of pregnanediol sulphate in liquor amnii.

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ISOLATION AND IDENTIFICATION OF 3α-HYDROXY-5α-ANDROST-16-ENE AND 5α-ANDROST-16-EN-3-ONE FROM PORCINE ADIPOSE TISSUE*

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(*Received* 19 *April* 1971)

3α-Hydroxy-5α-androst-16-ene and 5α-androst-16-en-3-one were isolated from the subcutaneous adipose tissue of swine and identified (Fig. 1*a*, *b*). These steroids, when excreted or secreted, could function as sex pheromones and initiate the olfactory communication stimulus regulating the mating behaviour of the domestic pig (*Sus domesticus*) (Signoret & du Buisson, 1961). Other researchers (Lerche, 1936; Craig, Pearson & Webb, 1962) have already established that a particularly strong sex odour (variously described as musk-, perspiration- and urine-like) was associated with the fatty tissues of the animal carcass. Even though the odoriferous, musk-smelling androstenols have been isolated from the testis (Prelog & Ruzicka, 1944), adrenal (Gower & Ahmad, 1966) and salivary glands (Patterson, 1968*b*) of the pig, they have not been identified as components of fatty or adipose tissue. Stimulated by our theoretical prediction (Sink, 1967) that 3α-hydroxy-5α-androst-16-ene should be present, and Patterson's subsequent finding (Patterson, 1968*a*) of the oxidized form, 5α-androst-16-en-3-one, we proposed to isolate the unique androstenol and androstenone from the subcutaneous adipose tissue (panniculus adiposus) of the male (boar) domestic pig (*Sus domesticus*). Two different approaches were employed.

First, an attempt was made to remove volatile substances from whole tissue samples by distillation at high temperature (150 °C) and atmospheric pressure, followed by trapping in liquid nitrogen. These conditions approximate to those currently used in the preparation of meat products from pork carcasses. Gas–liquid chromatographic (GLC) analyses of the ether-soluble volatile substances were performed using a 10% UC W98 column (1·8 m × 3·2 mm) operating isothermally at 200 °C. Our F & M Model 5755A instrument was equipped with a column effluent-splitter giving an approximate 8:1 partition between the heated collection vent and the flame ionization detector. This arrangement permitted olfactory analysis and trapping of the compounds as they emerged from the collection vent while the peak presentation was constantly monitored. Subsequent analyses using the LKB mass spectrometer confirmed the presence of the perspiration-like, urine-smelling 5α-androst-16-en-3-one but not the 3α-hydroxy-5α-androst-16-ene. This identification was facilitated by using androstenone and androstenol standards. Our observations agree with those of Patterson (1968*a*), namely, the presence of the androstenone

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but the absence of the androstenol in volatile substances from porcine adipose tissue.

Secondly, adipose tissue samples were extracted by heating at 70 °C, filtered, and the liquid fat saponified. The non-saponifiable lipids were taken up in benzene and partitioned against aqueous NaOH to separate the benzene-soluble neutral and basic components from the sodium salts of the acids and phenols. The former group was reacted with Girard T reagent (Talbot, Butler & MacLachlan, 1940)

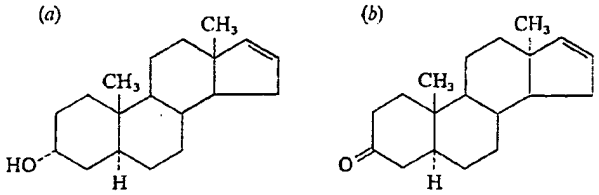

Fig. 1. The chemical structure of (*a*) 3α-hydroxy-5α-androst-16-ene, and (*b*) 5α-androst-16-en-3-one.

to prepare water-soluble derivatives of the oxosteroids and facilitate their separation from the non-ketonic, ether-soluble, hydroxysteroids. After the decomposition of the Girard T complexes, both fractions were subjected to GLC analyses. The odour of musk-smelling compounds predominated in the non-ketonic fraction, while the ketonic fraction was dominated by a perspiration- or urine-like odour. Mass spectral analyses confirmed the presence of both 3α-hydroxy-5α-androst-16-ene and 5α-androst-16-en-3-one. These observations confirm our earlier prediction (Sink, 1967) and Patterson's observations (1968*a*), support the olfactory observations of Craig *et al.* (1962) that sex odour compounds are present in the non-saponifiable fraction of the fat, and conclusively report for the first time the presence of both an androstenol and an androstenone in the subcutaneous adipose tissue of male pigs. Research is now underway to investigate the sex pheromone aspect of these compounds.

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ENDOGENOUS STEROID SULPHATES AND GLUCURONIDES IN THE GALLBLADDER BILE FROM EARLY AND MID-TERM HUMAN FOETUSES

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SUMMARY

The neutral steroids derived from their conjugates present in a pool of bile from 20 human foetuses were analysed by gas–liquid chromatography and gas chromatography–mass spectrometry. Ten monosulphates, twelve disulphates and seven glucuronides were detected, but no free steroids were found. Both saturated and unsaturated steroids of the C_{19}- and C_{21}-series were detected. The glucuronides of 5β-pregnane-3α,20α-diol, 5α-pregnane-3β,16α,20α-triol and 3α-hydroxy-5β-pregnan-20-one as well as the monosulphate of 16α-hydroxydehydroepiandrosterone were present in the highest concentrations. Other major components were the monosulphates of dehydroepiandrosterone, pregnenolone and 16α-hydroxypregnenolone and the disulphates of 5-androstene-3β,17α-diol, 3β,17β-dihydroxy-5-androsten-16-one and 5β-prenane-3ξ,20α,21-triol. The total concentration of the monosulphate conjugates was 435 μg/100 g sample (wet weight), of the disulphates 363 μg/100 g and of the glucuronides 815 μg/100 g.

Many of the compounds detected have previously been found in the foetal liver and intestinal contents. It is therefore concluded that during early and mid-gestation the foetal liver is already excreting many steroid conjugates through the biliary tract into the intestinal canal. As in other foetal compartments, considerable amounts of steroid sulphates were detected. However, the presence of steroid glucuronides in the bile suggests that the formation of hepatic glucuronides already occurs during the foetal period.

INTRODUCTION

It is known that the human foetus begins to secrete bile during the 12th week of gestation (Willis, 1962), and that in the human adult, a great variety of conjugates of neutral (Laatikainen, 1970*a*) and phenolic (Adlercreutz, 1962) steroids is secreted into the bile. The biliary secretion of steroids in the foetus has not been established but this seems likely, since many typical hepatic steroid metabolites have been detected in the intestinal contents of the foetus and newborn (Gustafsson & Stenberg, 1971; Kinsella & Francis, 1971; Huhtaniemi & Vihko, 1973). The compounds in the

bile may be either true hepatic steroid metabolites or metabolites formed in other parts of the foetoplacental unit. In addition, a part of these steroids may be involved in an enterohepatic circulation.

MATERIALS AND METHODS

Biological material

Human early and mid-term foetuses were obtained at interruption of pregnancy for socio-medical reasons. The foetus was delivered by abdominal hysterotomy. The gallbladder was excised, weighed, placed in acetone:ethanol (1:1, v/v) and stored at −20° C until analysed. Because of small sizes of the gallbladders, bile could not be separated from the gallbladder tissue. Twenty such samples were pooled and the pool analysed. The crown–rump lengths of the foetuses used varied from 9·0 to 18·0 cm and corresponded to gestational ages of 12 to 19 weeks (Tanimura, Nelson, Hollingsworth & Shepard, 1971). The pooled gallbladders weighed 2·45 g and the weights of the individual gallbladders varied from 10 to 670 mg.

Solvents and reference steroids were as described previously (Huhtaniemi & Vihko, 1973).

Trivial and systematic names

Androstenedione, 4-androstene-3,17-dione; androsterone, 3α-hydroxy-5α-androstan-17-one; testosterone, 17β-hydroxy-4-androsten-3-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; 16α-hydroxydehydroepiandrosterone, 3β,16α-dihydroxy-5-androsten-17-one; 16β-hydroxydehydroepiandrosterone, 3β,16β-dihydroxy-5-androsten-17-one; progesterone, 4-pregnene-3,20-dione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; 16α-hydroxypregnenolone, 3β,16α-dihydroxy-5-pregnen-20-one; 17α-hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one; 21-hydroxypregnenolone, 3β,21-dihydroxy-5-pregnen-20-one; stigmasterol, (24*S*)-24-ethylcholesta-5,22-dien-3β-ol.

Thin-layer chromatograms (t.l.c.) were developed in ethyl acetate–cyclohexane (1:1, v/v) as described previously (Laatikainen, 1970*b*).

20β-Hydroxysteroid dehydrogenase was used for the detection of 20β-hydroxyl groups essentially as described by Henning & Zander (1962).

For gas–liquid chromatography (g.l.c.) 3% QF-1 and 2·2% SE-30 columns and flame ionization detection were used, as described by Vihko, (1966) and Sjövall & Vihko (1966).

Gas chromatography–mass spectrometry (GC–MS) was carried out on an LKB 9000 instrument (LKB-Produkter AB, Stockholm-Bromma, Sweden) using QF-1 and SE-30 columns (Vihko, 1966; Sjövall & Vihko, 1966). The energy of the bombarding electrons was 70 eV and the ionising current 60 μA.

Procedure

A detailed description of the analytical procedure has been published elsewhere (Huhtaniemi, Luukkainen & Vihko, 1970*a*; Huhtaniemi & Vihko, 1972, 1973). In short, it was as follows: after homogenization and extraction with acetone:ethanol (1:1, v/v), the extract was applied to a 20 g Sephadex LH-20 column and fractionated into free steroids, their glucuronides, monosulphates and disulphates. The fraction of

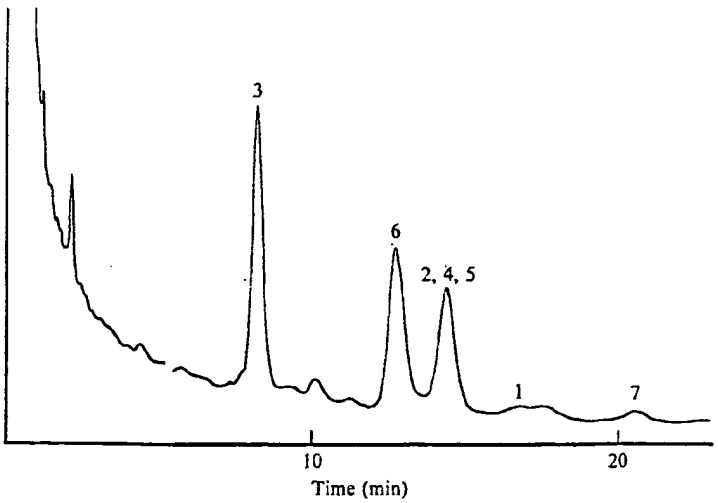

Fig. 1. Gas chromatographic analysis of neutral steroid trimethylsilyl (TMS) ethers derived from the glucuronide fraction of human foetal gallbladder bile, using a QF-1 column, 215 °C. The numbers refer to the TMS ether derivatives of (1) pregnenolone; (2) 3α-hydroxy-5β-pregnan-20-one; (3) 5β-pregnane-3α,20α-diol; (4) 3α,16α-dihydroxy-5α-pregnan-20-one; (5) 3β,16α-dihydroxy-5β-pregnan-20-one; (6) 5α-pregnane-3β,16α,20α-triol; (7) 3ξ,16ξ,19-trihydroxy-5ξ-pregnan-20-one.

unconjugated compounds was purified by partition between ethyl acetate and 0·1 M-NaOH. The purification and cleavage of the glucuronides with β-glucuronidase was performed as described by Laatikainen & Vihko (1969). The sulphates were solvolysed in ethyl acetate acidified with sulphuric acid. The solvolysed material was chromatographed on 3 g silicic acid columns and the final purification of each fraction was carried out on a 200 mg silicic acid column. The compounds were analysed by g.l.c. and by GC–MS as their trimethylsilyl (TMS) ether derivatives.

For quantitative analysis, a known amount of internal standard (10 μg stigmasterol) was added before the TMS ether derivative formation.

RESULTS

Table 1 summarizes our findings. The limit of detection was about 0·1 μg. This corresponds to a concentration of 5 μg steroid/100 g sample (wet weight). Ten monosulphates, twelve disulphates and seven glucuronides were found. The g.l.c. separation of the steroids (as their TMS ethers) derived from the glucuronide fraction is shown in Fig. 1.

Monosulphates

With the exception of androsterone, all the steroids detected in this fraction (Table 1) have recently been found in human foetal intestinal contents (Huhtaniemi & Vihko, 1973) and the details of their identification have been described in this connection. Androsterone was identified by its relative retention times (RRT values) on two liquid phases (see Materials and Methods) and its typical mass spectrum

Table 1. *Concentration of neutral steroids in the monosulphate, disulphate and glucuronide fractions in pooled human foetal gallbladders*

(The values are expressed as μg free steroid/100 g wet tissue and are uncorrected for methodological losses.)

Monosulphates	
Androsterone	Trace
Dehydroepiandrosterone	48
16α-Hydroxydehydroepiandrosterone	149
5-Androstene-3β,16α,17β-triol	29
Pregnenolone	47
3β-Hydroxy-5,16-pregnadien-20-one	23
5-Pregnene-3β,20α-diol	29
16α-Hydroxypregnenolone	52
17α-Hydroxypregnenolone	22
3ξ,15ξ,16ξ-Trihydroxy-5ξ-pregnan-20-one*	36
Total monosulphates	435
Disulphates	
5-Androstene-3β,17α-diol	51
5-Androstene-3β,17β-diol	16
16α-Hydroxydehydroepiandrosterone	28
16β-Hydroxydehydroepiandrosterone	26
3β,17β-Dihydroxy-5-androsten-16-one	54
5-Androstene-3β,16α,17β-triol	4
5α-Pregnane-3α,20α-diol and/or its 3β,5β-isomer	41
5β-Pregnane-3α,20α-diol	36
5-Pregnene-3β,20α-diol	28
16α-Hydroxypregnenolone	13
5β-Pregnane-3ξ,20α,21-triol	50
5-Pregnene-3β,20α,21-triol	16
Total disulphates	363
Glucuronides	
3α-Hydroxy-5β-pregnan-20-one	128
Pregnenolone	6
5β-Pregnane-3α,20α-diol	328
3α,16α-Dihydroxy-5α-pregnan-20-one / 3β,16α-Dihydroxy-5β-pregnan-20-one †	72
5α-Pregnane-3β,16α,20α-triol	262
3ξ,16ξ,19-Trihydroxy-5ξ-pregnan-20-one*	19
Total glucuronides	815

* Tentative identification.
† The individual concentrations of these compounds could not be determined by g.l.c. (see Results).

(Vihko, 1966). Another compound was identified tentatively as 3ξ,15ξ,16ξ-trihydroxy-5ξ-pregnan-20-one by its RRT values and mass spectrum (Gustafsson & Stenberg, 1971; Huhtaniemi & Vihko, 1973).

Disulphates

These compounds (Table 1) have also been detected recently in foetal intestinal contents (Huhtaniemi & Vihko, 1973). Because of very similar RRT values and mass spectra of 5α-pregnane-3α,20α-diol TMS ether and its 3β/5β-isomer (Laatikainen, Peltokallio & Vihko, 1968) the two compounds could not be distinguished. The low concentration of this chromatographic fraction prevented its rigorous identification. For the same reason, the orientation of the 3-hydroxyl group in 5β-pregnane-3ξ,20α,-21-triol remains unknown.

Glucuronides

Seven compounds were detected in this fraction (Table 1). Pregnenolone, 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3α,20α-diol were identified according to their RRT values and mass spectra (see Huhtaniemi & Vihko, 1973).

A fraction with RRT values of 1·57 and 0·96 on QF-1 and SE-30 columns, respectively, gave a mass spectrum typical of a 3,16-dihydroxypregnan-20-one TMS ether (Jänne & Vihko, 1970*a*). On t.l.c. (see Materials and Methods) this fraction was resolved into two bands. Comparison with authentic standards showed one to have a mobility identical with that of 3α,16α-dihydroxy-5α-pregnan-20-one (R_F value 0·24) and the other with that of 3β,16α-dihydroxy-5β-pregnan-20-one (R_F value 0·31). A compound with RRT values of 1·39 and 1·74 on the QF-1 and SE-30 columns, respectively, had a mass spectrum typical of a pregnane-3,16,20-triol TMS ether derivative. These RRT values were identical with those of authentic 5α-pregnane-3β,16α,20α-triol TMS ether. Another isomer with very similar RRT values, 5β-pregnane-3β,16β,20β-triol (Gustafsson & Stenberg, 1971) was excluded by treatment of the unknown compound with 20β-hydroxysteroid dehydrogenase (see Materials and Methods). No oxidation of the 20-hydroxyl group was observed. It was therefore concluded that this compound was 5α-pregnane-3β,16α,20α-triol. One compound in the glucuronide fraction was tentatively identified as 3ξ,16ξ,19-trihydroxy-5ξ-pregnan-20-one. Its RRT values and mass spectra were very similar to those of a compound previously detected in foetal and newborn meconium (Gustafsson & Stenberg, 1971; Huhtaniemi & Vihko, 1973). Furthermore, a compound with a mass spectrum reminiscent of a dihydroxy-mono-oxo-pregnane and another reminiscent of a trihydroxypregnane were found in the glucuronide fraction but their low concentrations did not allow further characterization.

Measurements

The quantity of each neutral steroid sulphate and glucuronide conjugate detected is shown in Table 1. The accuracy of the present method has been discussed earlier in reference to some foetal tissues (Huhtaniemi *et al.* 1970*a*) and adult human bile (Laatikainen, 1970*a*) by determining the recoveries of several steroid sulphate and glucuronide standards added to the sample to be analysed. The mean recoveries were about 70–100%. The values in Table 1 have not been corrected for analytical losses.

DISCUSSION

Bile is formed in the human foetus from the 12th week of gestation (Willis, 1962), and from the results of the present study it would seem that the bile formed during early and mid-gestation already contains many neutral steroids in high concentrations. As in adult bile (Laatikainen, 1970*a*) these compounds are present as their sulphates and glucuronides. No free steroids were present in concentrations greater than the limit of detection (see Results).

Some errors may have arisen as a result of the sample's heterogeneity, since it was impossible to separate the small gallbladder tissue from its contents. If one assumes that the steroid concentration in the gallbladder tissue is very low, the values given in Table 1 would be considerably lower than those for the bile alone.

Human foetal tissues have a high sulphate-conjugating capacity (Wengle, 1966) and so far, most of the endogenous neutral steroids detected in foetal tissues have been in a sulphate conjugated form (see Huhtaniemi & Vihko, 1973). Therefore it was expected that the neutral steroids in the bile would also be present as sulphates. As in the case of the foetal intestinal contents (Huhtaniemi & Vihko, 1973), about half of the sulphated compounds detected were mono- and half were disulphates. In foetal liver tissue (Huhtaniemi *et al.* 1970*a*), on the other hand, no disulphates were detected, which might indicate a rapid turnover of steroid disulphates in this tissue.

The glucuronide conjugating capacity of foetal tissues has been reported to be very low (Dutton, 1959). However, some glucuronides of neutral steroids have been detected in the foetal meconium (Gustafsson & Stenberg, 1971; Kinsella & Francis, 1971), in cord blood from the newborn (Eberlein, 1965) and in amniotic fluid at term (Schweitzer, Klein & Giroud, 1971). The results of incubation and perfusion studies suggest that the liver and gastrointestinal tract are the most active sites of foetal glucuronide conjugation (Mikhail, Wiqvist & Diczfalusy, 1963; Wu, Archer, Flickinger & Touchstone, 1970). Glucuronide conjugating activity has also been demonstrated in the placenta (Smith & Axelrod, 1969) and in several other foetal tissues (Ducharme, Limal, Antic & Sandor, 1973). Thus, the glucuronides detected in the bile might well be foetal metabolites. The transplacental passage of these compounds from the maternal side cannot, however, be excluded since perfusion studies with oestrogen glucuronides have demonstrated transfer across the placenta without hydrolysis (Diczfalusy, 1969). The formation of glucuronides in the foetal liver is strongly suggested by the observation that pregnenolone, 3α-hydroxy-5β-pregnan-20-one, 5β-pregnane-3α,20α-diol and 3β,16α-dihydroxy-5β-pregnan-20-one, the neutral steroids previously detected in this tissue in an unconjugated form (Huhtaniemi, 1973), were all present as glucuronides in the bile. Since the liver does not contain detectable amounts of glucuronides (I. Huhtaniemi, unpublished observation) this would suggest a rapid elimination of these compounds upon conjugation. Of the compounds detected in this study of foetal bile the majority have previously been identified as components of meconium (Huhtaniemi & Vihko, 1973). These findings would suggest the bile as an important source of foetal intestinal steroids. Another possible source of these steroids is the amniotic fluid swallowed by the foetus. The most striking difference between the steroid pattern in the bile and the intestinal contents is the lack of steroids with a 21-hydroxy-20-oxo side-chain (e.g. 21-hydroxypregnenolone) in the bile. Several such compounds have been detected in the amniotic fluid (Jänne & Vihko, 1970*b*) which suggests that these steroids enter the intestinal tract from the swallowed amniotic fluid.

Many of the steroids identified in this study contain a 3β-hydroxy-5-ene structural fragment which is quite typical of foetal steroid metabolites (Diczfalusy, 1969). Also many of the steroids detected were 16-hydroxylated compounds, which is considered characteristic of foetal liver steroid metabolites (Diczfalusy, 1969). Similarly, all the steroids of the pregnane series are most probably products of the foetal hepatic metabolism of placental progesterone.

Of the steroids detected in this study, androsterone is the only one which has not been previously detected as an endogenous foetal steroid. However, its presence in the foetal liver has been demonstrated after whole foetal perfusion with androstene-

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dione and testosterone (Benagiano, Mancuso, Mancuso, Wiqvist & Diczfalusy, 1968), steroids which have been detected in the foetal testes (Huhtaniemi, Ikonen & Vihko, 1970*b*).

This study demonstrates that a great variety of sulphates and glucuronides of neutral steroids pass from the foetal liver into the biliary tract. The conjugation of neutral steroids with glucuronic acid seems also to take place in the foetus, probably in the foetal liver. The rate of formation of bile in the foetus is not known, and thus the quantitative importance of this biliary steroid secretion remains to be established. The steroids detected in the foetal bile pool may be the products of active steroid metabolism in the liver or other foetal tissues or may reflect the accumulation of some minor components of the enterohepatic circulation.

I wish to thank Professor R. Vihko, M.D., for his interest during the course of this work. The skilful technical assistance of Mrs Kerttu Mårtensson is gratefully acknowledged.

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Thank you

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THE MEASUREMENT OF 3β-HYDROXY-Δ^5 STEROIDS IN HUMAN FETAL BLOOD, AMNIOTIC FLUID, INFANT URINE AND ADULT URINE

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ABSTRACT

A relatively simple new technique is reported for the separation and measurement of the following Δ^5 steroids in human fetal blood, amniotic fluid and infant and adult urine: 3β-hydroxypregn-5-en-20-one, 3β-hydroxyandrost-5-en-17-one, 3β,21,-dihydroxypregn-5-en-20-one, 3β,17α(and 17β)-dihydroxyandrost-5-ene, 3β,17β-dihydroxyandrost-5-en-16-one, 3β, 16α-dihydroxyandrost-5-en-17-one, 3β,16α-dihydroxypregn-5-en-20-one, 3β,16α,17β-trihydroxy-androst-5-ene and two unidentified compounds. After enzyme hydrolysis and solvolysis the steroids are extracted from the biological material and separated on thin-layer silica-gel chromatograms. Assay is by direct photo-electric scanning of the coloured bands developed on the chromatograms by an antimony trichloride reagent. The identity of the compounds has been checked by gas chromatography-mass spectroscopy and further thin-layer chromatography. The accuracy, specificity and precision of the method have been evaluated. The total urinary excretion of the compounds when expressed in μg/24 hr/m^2 body area is approximately 10 times that of the adult. The 2 unknown compounds, 21-hydroxypregnenolone and 16-hydroxypregnenolone are undetectable in adult urine. In cases of the adrenogenital syndrome the excretion of 16-hydroxypregnenolone was markedly increased.

INTRODUCTION

Steroids with the Δ^5-3β-hydroxy configuration predominate quantitatively and qualitatively over all other steroids (cholester 1 apart) in fetal blood and infant urine. In adults the quantitative importance of such steroids is largely limited to dehydroepiandrosterone (DHA)[1], but in the fetus and in infants a large number of other Δ^5 steroids and Δ^5 steroid-like compounds are present, some in considerable quantity (1 - 3).

Easterling et al. (4) have shown that the concentration of DHA-sulfate and 16α-hydroxy-DHA-sulfate in fetal blood totals 200 - 300 μg/100 ml. and Reynolds (5) has recorded a total urinary excretion of 16α-hydroxypregnenolone (16-OH-Preg.), 16α-hydroxy-DHA (16-OH-DHA) and androstenetriol (AT) of 14 mg./24 hr. in a 3 week old premature infant and of over 8 mg./24 hr. in 6 similar infants aged up to 5 weeks.

It is only comparatively recently that the importance of the Δ^5 steroids in early life has been appreciated and the techniques of assay so far used have been tedious, most of them having been designed more to achieve the isolation of pure compounds rather than for assay purposes (1,3-8). It is apparent that much is to be gained from a quantitative study of the individual Δ^5 steroids circulating in the feto-placental unit and excreted by the infant during the first few months of independent life (9); also there is an immediate clinical application in that an early diagnosis of any dysfunction in adrenocortical steroid synthesis may depend upon an ability to measure

[1] Abbreviations for compounds used throughout the text are shown in Table 1.

accurately the Δ^5 steroids in day-old infant urine (10). In adults it is not usually considered worthwhile to investigate the 3β-hydroxy-Δ^5 steroids as a group but as they constitute by far the most abundant group in infants, it is of considerable importance that more should be known of their excretion in the normal and abnormal infant.

During the development of a suitable technique, an attempt has been made to balance specificity, sensitivity and reliability against

ase of operation and the p ssibility of measuring the individual steroids in a urine sample from a day-old infant, and in th small samples of arterial and venous cord blood which can be obtained from single umbilical cords. The compounds chosen for assay are shown in Table 1 and include two chromatographic bands of material staining with antimony trichloride which have not been identified, but they are probably steroidal and if so, are of considerable quantitative significance.

The technique has been used to measure the excretion of urinary Δ^5 steroids during the first few days of infant life, and the rates of excretion are compared with those found in adults. Findings in 2 cases of the adrenogenital syndrome are also reported.

MATERIALS

All the reagents used were of analytical grade unless otherwise specified. Other material used was obtained as follows:-

Succus entericus from the snail Helix pomatia - L'Industrie Biologique S.A., Gennevilliers, France.

Silica-gel, grade Hf 254 - Merck A.G., Darmstadt, Germany.

Thin-layer chromatography spreader - Quickfit and Quartz Ltd., Stone, England.

Thin-layer chromatography tanks - Shandon Scientific Co. Ltd., London, England.

Thin-layer chromatogram scanner - Joyce, Loebl and Co. Ltd., Gateshead 11, England.

Gas chromatograph-mass spectrograph - LKB 9000. LKB Produkter AB, Stockholm, Sweden.

The source of steroids used as authentic standards is indicated in Table 1.

Complete 24 hr. specimens of urine were collected from different normal infants on each day for the first 6 days of life, and also from normal adult males. Preservation was by storage at 4° followed by freezing at -24° as soon as possible. Umbilical cord blood was heparinized and the plasma separated as soon as possible. Plasma and amniotic fluid were stored at -24°.

METHOD

Choice of Technique

To ensure the extraction and measurement of as much steroid material as possible a comprehensive hydrolysis procedure was chosen, and for separating the steroids in the extract, thin-layer was decided upon in preference to column, paper or gas-liquid chromatography. The quantities of steroid being dealt with, particularly in blood, were small and the assay of bands of material on paper or thin-layer chromatograms was preferred to the use of column chromatography involving the collection of many eluate fractions. Paper chromatography was found to necessitate a complex preliminary purification stage (6), long development periods were required and the spots or bands of steroid formed tended to be more diffuse than those obtained using thin-layer chromatography. Many of the compounds were found to be unstable when subjected to gas-liquid chromatography.

Elution of the bands of material from the thin-layer plates, with subsequent colorimetric assay did not prove practicable because many were too close together for accurate detection and delineation using a parallel run of standards. Assay was therefore achieved by direct staining and quantitative scanning.

Preliminary studies

Hydrolysis. Leon et al. (11) have shown that the crop fluid of the snail Helix pomatia contains both β-glucuronidase and sulfatases. Eberlein (1) has demonstrated that the Δ^5-3β-OH steroids are present in cord blood mainly in sulfated form, and it was found that the use of the Helix pomatia enzyme alone did not achieve complete hydrolysis of the Δ^5 steroids present in the biological fluids studied. A second stage involving a solvolytic procedure (12) was essential.

To test the completeness of hydrolysis by the two-stage process, the residual hydrolysate after extraction with ether:ethyl-acetate was further extracted with butanol to remove any material still conjugated. The butanol extract was evaporated to dryness, taken up in water and the two stage hydrolysis repeated. This procedure was carried out 6 times on infant urine and no detectable spots were produced after spraying the chromatograms finally produced from the extracts.

TABLE 1

Compounds assayed, together with their abbreviations, trivial names and the source of origin of the standards used. (Given in order of increasing polarity).

Pregnenolone (Preg)	3β-hydroxypregn-5-en-20-one.[a]
Dehydroepiandrosterone (DHA)	3β-hydroxyandrost-5-en-17-one.[a]
U_1	Unknown $SbCl_3$ staining compound.
21-Hydroxypregnenolone (21-OH-Preg)	3β,21-dihydroxypregn-5-en-20-one[b]
Androstenediol (AD)	3β,17α(and 17β)-dihydroxyandrost-5-en[b]
16-oxoandrostenediol (16-O-AD)	3β,17β-dihydroxyandrost-5-16-one[c]
16α-Hydroxydehydroepiandrosterone (16-OH-DHA)	3β,16α-dihydroxyandrost-5-en-17-one[d]
U_2	Unknown $SbCl_3$ and blue tetrazolium staining compound
16α-Hydroxypregnenolone (16-OH-Preg)	3β,16α-dihydroxypregn-5-en-20-one[b]
Pregnenetriol	3β,17α,20α-trihydroxypregn-5-en[b]
Pregnanetriol	3α,17α,20α-trihydroxy-5β-pregnan[b]
Androstenetriol (AT)	3β,16α,17β-trihydroxyandrost-5-en[c]

(a) Koch-Light Laboratories Ltd., Colnbrook, England.

(b) Medical Research Council Reference Collection.

(c) Synthesized by P.J. Sykes and R.W. Kelly.

(d) Drs. D. Fukushima, P.J. Sykes and R.W. Kelly.

Preliminary purification. With the thin-layer chromatography systems used, it was found that the ether:ethyl acetate extract of urine and the ethanol:ether (see later) extracts of amniotic fluid and blood, needed only alkali, mild acid and water washes.

Thin-layer Chromatography. In order to achieve adequate separation of the antimony trichloride (see later) staining compounds which appear to be important in the biological materials studied, a

procedure of chromatogram devel pment using multiple solvent runs was designed using the same or different solvent systems. Multiple running with systems giving low Rf values was found to give better separations than the more normally used single stage development procedure. In all cases plates were prepared using silica-gel and the following solvent systems were selected for use:-

System A - Chloroform
System B - Chloroform : absolute ethanol (95:5 v/v)
System C - Chloroform : absolute ethanol (90:10 v/v)
System D - Benzene : absolute ethanol (96:4 v/v)
System E - Cyclohexane : ethyl acetate (50:50 v/v)

Compounds which proved difficult to separate either from each other or from interfering chromatogenic material were:-

1. DHA from Preg
2. U_1 from 21-OH-Preg
3. 16-O-AD from 16-OH-DHA
4. AT

The use of System D will satisfactorily separate the substances listed under 1 and 3 but not those under 2, separation of these may however be achieved by using System A. The desired properties of the two systems may be combined by running the plates three times in System A then three times in System D; the resulting separation then produced for infant urine is shown in Fig. 1.

Since AT is considerable more polar than the other compounds to be assayed, when adequate distribution of the main group is obtained AT moves only a short distance from the origin and is poorly separated from neighbouring substances. A second chromatogram must therefore be prepared and run in the more polar solvent system C.

Fig. 2 shows the separation achieved for infant urine after two runs in this system. It will be seen that pregnanetriol is also separated, staining as a yellow band. Quantitative information on the content of this steroid in infant urine can be of considerable diagnostic importance in addition to the measurement of the Δ^5 steroids, in cases of the adrenogenital syndrome.

In adult urine there is qualitatively and quantitatively less Δ^5 steroid present, and unlike infant urine, pregnenetriol assumes quantitative importance, but in the systems designed for infants an unidentified band staining with antimony trichloride is not separated from pregnenetriol. For adults therefore, the problems of separation are different but less complex and a procedure utilizing a single chromatogram has been chosen.

The plates are run once in System E, then twice in System B and it will be seen in Fig. 3 that AT and pregnanetriol move sufficiently far from the origin to be separated and measured, but 16-OH-DHA and 16-O-AD are not separated. This disadvantage may easily be overcome if necessary, by carrying out additional runs in the systems designed for infants.

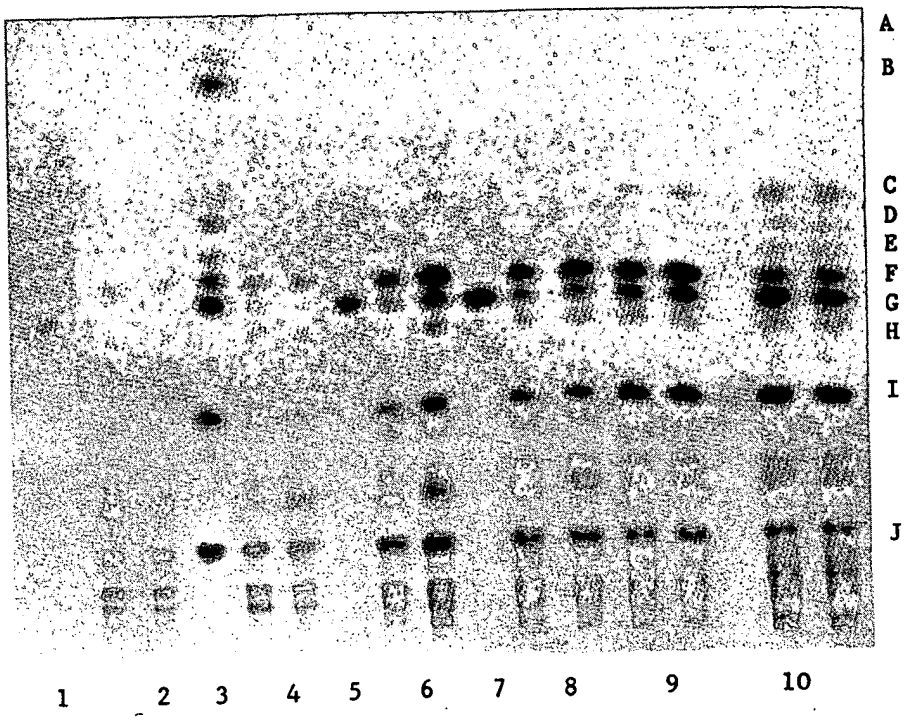


Figure 1. A typical 20 x 20 cm. thin-layer chromatography plate for the assay of the following compounds in infant urine: A, pregnenolone; B, DHA; C, unknown 1; D, 21-OH-pregnenolone; E, androstenediol; F, 16-oxo-androstenediol; G, 16-OH-DHA; H, unknown 2; I, 16-OH-pregnenolone. The position of androstenetriol (not assayed) is shown at J. Samples of 16-OH-DHA were run in positions 1 (1.25 μg), 5 (3.75 μg) and 7 (5.0 μg). Samples (2.5 μg) of all the steroids measured (unknown compounds apart) were run in position 3. Duplicate extracts of urines collected from the same baby on days 1, 2, 3, 4, 5 and 6 of life were run in positions 2, 4, 6, 8, 9 and 10. The plate was sprayed with antimony trichloride followed by heating.

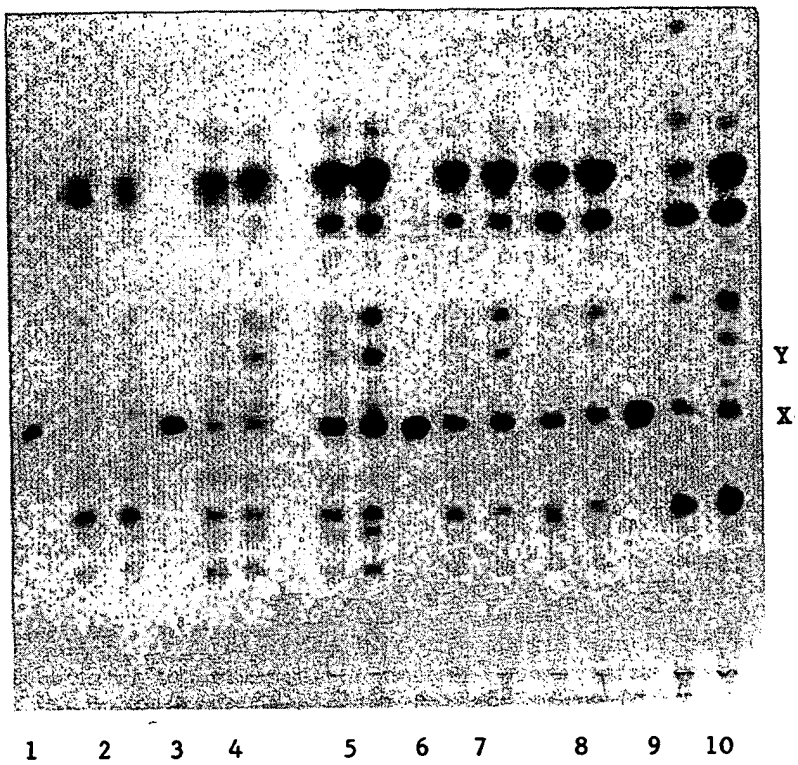
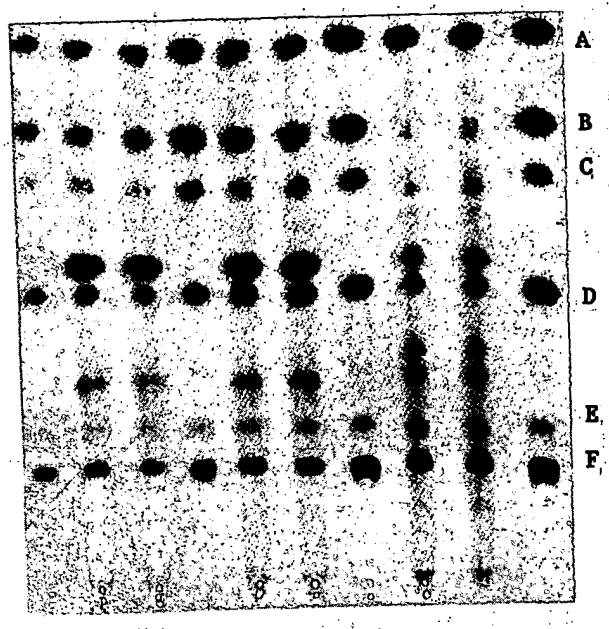


Figure 2. A typical 20 x 20 cm. thin-layer chromatography plate for the assay of androstenetriol (X) in infant urine. The position of pregnanetriol, if present, is shown at Y. Standard samples of androstenetriol were run in positions 1 (1.25 μg), 3 (2.50 μg), 6 (3.75 μg) and 9 (5.0 μg). Urine extracts run in duplicate in positions 2, 4, 5, 7, 8 and 10 were the same as shown in Fig. 1. The plate was sprayed with antimony trichloride followed by heating.



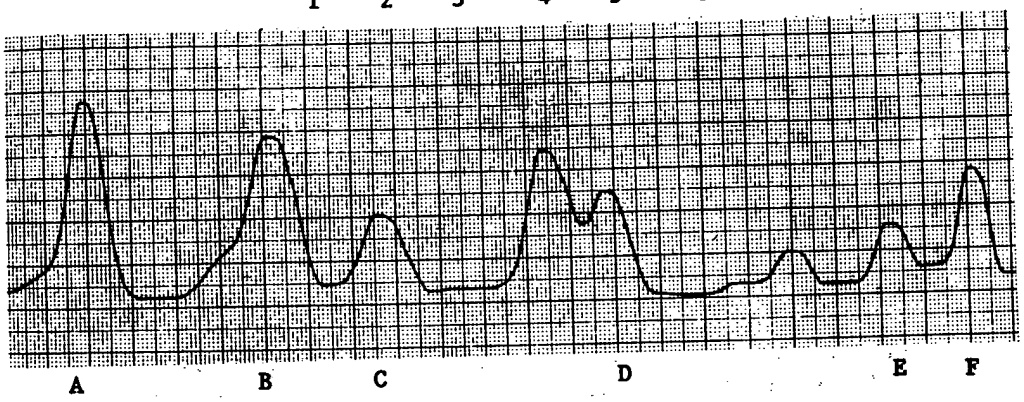


Figure 3. (Above). Part of a typical thin-layer chromatography plate for the assay of the following compounds in adult urine: A, DHA; B, androstenediol; C, 16-OH-DHA and 16-oxo-androstenediol; D, pregnenetriol; E, pregnanetriol; F, androstenetriol. Standard steroids were run in positions 1 (1.25 μg), 3 (2.50 μg), 5 (3.75 μg) and 7 (5.0 μg). Duplicate extracts of urines from 3 different adults were run in positions 2, 4 and 6. The plate wa sprayed with antimony trichloride followed by heating.

(Below). The recording produced by run 4 when scanned in a photoelectric reflectance densitometer.

Staining technique. Spraying the thin-layer chromatograms with antimony trichloride in chloroform solution followed by heating at 110° for 5 min. produces a red colour with Δ^5-3β-hydr xysteroids (13). This reaction was selected as being the most suitable both with regard to specificity and quantitation of the compounds to be investigated. It has previously been used for the quantitative assay of cholesteryl esters on silica-gel plates (14).

Details of the Method.

Hydrolysis and Extraction

1. Urine

(a) Hydrolysis
- (i) See Table 2 for the volumes to be taken.
- (ii) Adjust to pH 11.5 with 4N NaOH.
- (iii) To precipitate sulfate ions which may interfere with enzyme action add 10% $BaCl_2$ (w/v) until no further precipitation is obtained.
- (iv) Adjust to pH 5 with 50% HCl (v/v).
- (v) Add 10% of the volume of 5N acetate buffer (5N acetic acid, 5N sodium acetate, 2:3, v/v).
- (vi) Add 0.1 ml. of the succus entericus of Helix pomatia per 20 ml. urine. Incubate at 37° for 24 hr.
- (vii) Repeat (vi).

(b) Extraction
- (i) Extract twice with 2 volumes of ether:ethyl acetate (2:1, v/v).
- (ii) Wash the extract repeatedly with 10% of the volume of N NaOH until the washings are clear.
- (iii) Wash once with 10% of the volume of water with 2-3 drops of acetic acid added.
- (iv) Wash once with 10% of the volume of water.
- (v) Evaporate to dryness on a rotary evaporator at 45°.
- (vi) Transfer the extract with chloroform into a small tube, dry down under nitrogen.

(c) Solvolysis
- (i) Bring the aqueous phase from b (i) to pH 1 with 50% H_2SO_4 (v/v).
- (ii) Saturate with ammonium sulfate.
- (iii) Extract twice with 1 volume of ethyl acetate, incubate the extract at 38° for 24 hr.
- (iv) Repeat b (ii to vi). The solvolysis extract is combined with the enzyme freed extract in the same tube.

TABLE 2

V lumes of urine, extract etc. required at various stages in the technique.

	Infant urine	Adult urine	Amniotic fluid	Umbilical cord plasma
Volume normally taken	1/16 24 hr. spec.	1/60 24 hr. spec.	100 ml	10 ml
Volume of ethanol used for dissolving extract (µl)	500	160	140	100
Volume of ethanol sol. normally required per chromatogram spot (µl)				
Preg	100 - 200	-	50	50
DHA	100 - 200	20	50	50
U_1	10 - 40	-	20	50
21-OH-Preg	10 - 40	-	20	50
AD	10 - 40	20	20	50
16-O-AD	10 - 20	-	20	50
16-OH-DHA	10 - 20	20	20	50
U_2	10 - 20	-	20	50
16-OH-Preg	10 - 20	-	20	50
Pregnenetriol	-	20	-	-
Pregnanetriol	-	20	-	-
AT	10 - 20	20	40	50

2. Amniotic Fluid and Umbilical Cord Plasma

(i) See Table 2 for the volumes to be taken
(ii) Precipitate proteins with 4 volumes of ethanol, centrifuge.
(iii) Extract the precipitate with 100 ml. of 80% ethanol (v/v), centrifuge.
(iv) Combine the ethanolic solutions and evaporate to dryness on a rotary evaporator at 45°.
(v) Add 30 ml. of distilled water and extract twice with 30 ml. ether.

(vi) The extract contains the free steroids and because of fatty impurities it is not suitable for thin-layer chromatography and is normally discarded. If required for other purposes it may be further treated as under 1b (ii-vi).

(vii) Hydrolyse and extract the aqueous fraction from (v) as for urine. The final extract will contain the conjugated steroids.

Thin-layer Chromatography. Silica-gel is spread onto 20 x 20 cm plates to a thickness of 0.25 mm. No activation is required but the plates are allowed to stand for at least 24 hr. at room temperature before use. Extracts are "spotted" onto two prepared plates in the amounts shown in Table 2. Duplicate extracts of up to 6 urines may be run on each plate (see Figs. 1 - 3). Care must be taken to ensure that all the spots are as nearly as possible 0.5 cm diameter. The spot pairs are interspersed with spots containing 1.25, 2.50, 3.75 and 5.0 μg of 16-OH-DHA (for infant medium polarity compounds), AT (for infant high polarity compounds) and DHA and 16-OH-DHA (for adult Δ^5 compounds), to be used for quantitative calibration. One spot containing 2.5 μg of each steroid to be assayed is also prepared to check the quantitation of each steroid.

The plates thus prepared are placed momentarily in a highly polar development system (ethyl acetate:methanol, 3:1 v/v) to "run up" the spots approximately 0.5 cm, so that bands are produced instead of circles. This procedure is repeated a second time, allowing the plate to dry in between. Very narrow bands of material are thus produced, making it possible to achieve maximal chromatographic separation.

The solvent systems used for development have been described in a previous section. Multiple runs are carried out by drying the plates in air after development in one system, then immediately placing them for a further run either in the same or in another system. Details of development are as follows:

infant urine)	for medium polarity compounds	(3 x System A
cord plasma)		(3 x System D
amniotic fluid)	for AT	2 x System C
adult urine		(1 x System E
		(2 x System B

Colour Development. The fully developed plates are sprayed evenly with a saturated solution of antimony trichloride in chloroform and heated for 5 min. in an oven with an even temperature of 110°. The colours formed will fade after several hours in the open air but if the chromatograms are covered with a glass plate they may be kept successfully in the refrigerator for several days. It is however recommended that they be scanned as soon as possible.

Quantitation. Each developed chromatogram spot is scanned in succession in the thin-layer scanner using reflectance, a slit 1 x 7 mm and the no. 490 filter (max. transmission 490 mμ). The base-line is drawn in

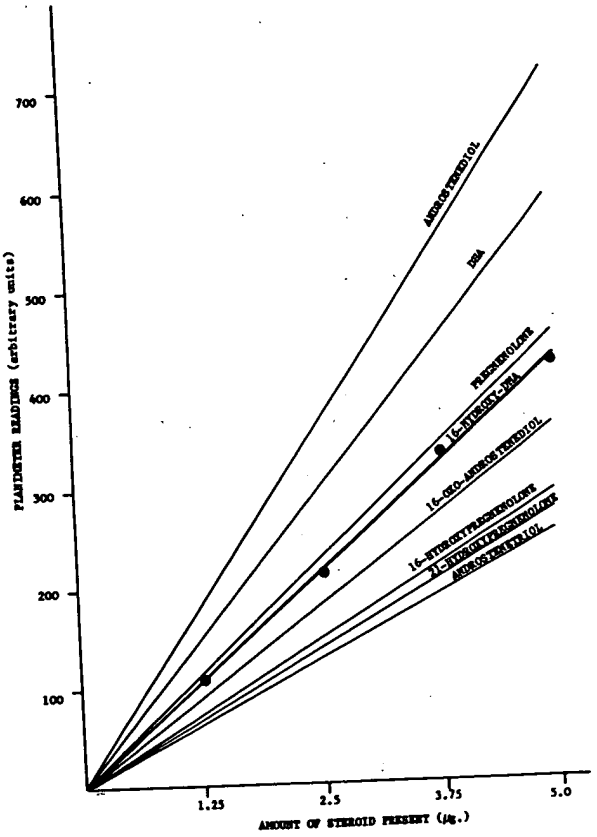


Figure 4. Calibration graphs for standard steroids run on thin-layer chromatograms, stained with antimony trichloride, scanned by a reflecting densitometer and the areas under the peaks measured by planimetry.

on the resulting recorder trace (Fig. 3) and perpendicular lines are inserted to divide each peak, areas are measured by planimetry. The integrator on the scanner is not used because no correction can then be made for a base-line which is often slightly sloping. A standard curve is constructed from the areas under the standard 16-OH-DHA peaks and similar graphs based on the single multistandard run are drawn for the other compounds assayed (Fig. 4). The graphs are then used for quantitation of the compounds in the extracts. (Greater precision may be obtained by using 4 multistandard runs but the above scheme is suggested as it is usually necessary to conserve valuable steroid standards).

RESULTS

Method Evaluation

Accuracy and Specificity. As a measure of accuracy (the concordance between the determination and the true value of the quantity measured), the recovery of DHA from DHA sulfate has been determined. DHA sulfate (250 μg) was added to 10 ml. of pooled 1 - 3 day old infant urine and after hydrolysis and extraction 1/50th of the extract was chromatographed and assayed. The average recovery for 6 experiments was 80% ± 6.2 (S.D.).

The antimony trichloride reaction used for colour development was thoroughly investigated to determine its specificity (the ability of a technique to determine solely the compound it purports to measure). The preliminary purification and separation stages of the method were chosen purposely for their simplicity and ease of operation, and consequently the substances to be assayed are not obtained on the chromatograms in pure form; the specificity of the colour reaction is therefore important.

The fluorescence producing reactions of antimony trichloride are very unspecific (15) but the reaction considered here produces in normal light, specific colours for certain groups of steroids when the amount of each steroid on the chromatogram is less than 10 μg.

Table 3 indicates the various colours which were found to be produced; some others have been reported previously (13, 16, 17). With the exception of the estrogens (removed in the preliminary alkali wash) most of the major biologically common groups of steroids are represented and it will be seen that all the 3β-OH-Δ^5 steroids

TABLE 3 Colours produced by the antimony trichloride reaction with various steroids on silica-gel thin-layer chromatograms

3β-Hydroxy-Δ^5 steroids

Androstenes

3β-Hydroxyandrost-5:16-dien	red
3β-Hydroxyandrost-5-en-17-one	red
3β-Hydroxyandrost-5-en-17-one	red
3β-Hydroxyandrost-5,15-dien-17-one	red
3β-Hydroxyandrost-5-en-11:17-dione	red
3β,16α-Dihydroxyandrost-5-en-17-one	red
3β,16β-Dihydroxyandrost-5-en-17-one	red
3β,17β-Dihydroxyandrost-5-en-11-one	red
3β,17β-Dihydroxyandrost-5-en-16-one	red
3β,17β-Dihydroxyandrost-5-en	red
3β,15α,17β-Trihydroxyandrost-5-en	red
3β,16α,17α-Trihydroxyandrost-5-en	red
3β,16α,17β-Trihydroxyandrost-5-en	red
3β,16β,17β-Trihydroxyandrost-5-en	pale blue-grey
3β,17α-Dihydroxyandrost-5-en	pale blue-grey
3β,17β-Dihydroxyandrost-5-en	dark blue-grey
1α,3β-Dihydroxyandrost-5-en-17-one	dark blue-grey
3β,11β-Dihydroxyandrost-5-en-17-one	dark blue-grey
3β,16α-Dihydroxyandrost-5-en-17-one	dark blue-grey
3β,11β,17β-Trihydroxyandrost-5-en	dark blue-grey
3β,16β,17β-Trihydroxyandrost-5-en	dark blue-grey
3β,15β,17β-Trihydroxyandrost-5-en-17-one	turquoise*
3β,7α-Dihydroxyandrost-5-en-17-one	turquoise*
3β,7β-Dihydroxyandrost-5-en-17-one	turquoise*
3β-Hydroxyandrost-5-en-7,17-dione	no colour

Other steroids

3-Oxo-Δ^4 steroids

Androst-4-en-3,17-dione	no colour
Androst-4-en-3,11,17-trione	no colour
Androst-4-en-3-one	no colour
17β-Hydroxyandrost-4-en-3-one	blue
17α-Hydroxyandrost-4-en-3-one	no colour
Pregn-4-en-3,20-dione	no colour
16α-Hydroxypregn-4-en-3,20-dione	no colour
17α-Hydroxypregn-4-en-3,20-dione	no colour
21-Hydroxypregn-4-en-3,20-dione	no colour
16α,21-Dihydroxypregn-4-en-3,20-dione	no colour
21-Hydroxypregn-4-en-3,11,20-trione	no colour
11β,21-Dihydroxypregn-4-en-3,20-dione	no colour
17α,21-Dihydroxypregn-4-en-3,20-dione	no colour
17α,21-Dihydroxy-pregn-4-en-3,11,20-trione	no colour
11β,17α,21-Trihydroxypregn-4-en-3,20-dione	no colour
6β,11β,17α,21-Tetrahydroxypregn-4-en-3,20-dione	no colour
Androst-1,4,dien-3,17-dione	no colour
Androst-1,4,dien-3-one	yellow
17β-Hydroxy-1,4,dien-3-one	no colour

Pregnenes

3β-Hydroxypregn-5-en-20-one	red
3β-Hydroxypregn-5,16-dien-20-one	red
3β,16α-Dihydroxypregn-5-en	red
3β,17α-Dihydroxypregn-5-en	red
3β,20β-Dihydroxypregn-5-en	red
3β,21-Dihydroxypregn-5-en	red
3β,17α-Dihydroxypregn-5-en 11,20 dione	red
3β,17α-Dihydroxypregn-5-en-20-one	red
3β,17α,21-Trihydroxypregn-5-en	red
3β,20β,21-Trihydroxypregn-5-en	blue-grey
3β,20α-Dihydroxypregn-5-en	blue-grey
3β,17α,20α-Trihydroxypregn-5-en	blue-grey
3β,17α,20β-Trihydroxypregn-5-en	blue-grey
3β,17α,20α,21-Tetrahydroxypregn-5-en	blue-grey

Cholestenes

Cholesterol	violet
7α-Hydroxy cholesterol	turquoise*
7β-Hydroxy cholesterol	turquoise*

Saturated steroids

3α,17α,21-Trihydroxy-5β-pregnan-20-one	no colour
3α,17α,21-Trihydroxy-5β-pregnan-11-20-dione	no colour
3α,17α,21-Trihydroxy-5α-pregnan-11-20-dione	no colour
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one	no colour
3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one	no colour
3α-Hydroxy-5β-androstan-17-one	no colour
3α-Hydroxy-5β-androstan-11,17-dione	no colour
3α,11β-Dihydroxy-5β-androstan-17-one	no colour
3α,11β-Dihydroxy-5α-androstan-17-one	no colour
3α-Hydroxy-5α-androstan-11,17-dione	no colour
3α,11β-Dihydroxy-5α-androstan-17-one	no colour
3β-Hydroxy-5α-androstan	yellow
3β,17β-Dihydroxy-5α-androstan	yellow
3α,17β-Dihydroxy-5β-androstan	yellow
3β-Hydroxy-5α-pregnan-20-one	yellow
3β,16α-Dihydroxy-5α-pregnan-20-one	yellow
3α,20α-Dihydroxy-5β-pregnan	yellow
3α,17α,20α-Trihydroxy-5β-pregnan	yellow
3α,17α,20α-Trihydroxy-5β-pregnan-11-one	yellow
3α,6α-Dihydroxy-5β-pregnan-20-one	orange

Miscellaneous

Androst-5-en-3,11,17 trione	no colour
3β-Hydroxycholest-4-en	red*
3β-Hydroxycholest-1-en	red*
Cholest-5-en-3-one	no colour

Chromatograms were developed with 10 μg. of steroid in each case.

* Immediate colour produced without heating.

give a strong colour except when a 7-oxo group is present. Compounds with a 7-OH group are readily distinguished by their producing an immediate turquoise colour without heating. None of the 3-oxo-Δ^4 steroids listed gives a colour except epitestosterone (17α-hydroxyandrost-4-en-3-one) which produces a blue colour, and a yellow colour is produced by a $\Delta^{1,4}$ steroid. Amongst the saturated steroids, only a 6α-OH steroid gave an orange colour, all others tested produce either a weak yellow colour or no colour at all. Pregnanetriol gives an intense yellow colour by which it may be assayed. If careful consideration is given to the data in Table 3 and chromatogram development systems are carefully chosen, the antimony trichloride reaction on thin-layer chromatograms can in practice be very specific.

Extracts from the biological materials under investigation were run firstly in one direction using the systems normally used, and then a second development was carried out at 90° to the first using the following thin-layer chromatography solvent systems:

chloroform: absolute ethanol (95:5, v/v)

ethyl acetate: water saturated with n-hexane:
glacial acetic acid: absolute ethanol (72:13.5:10:4.5, by vol.)

cyclohexane: ethyl acetate (50:50, v/v)

cyclohexane: ethyl acetate: absolute ethanol (45:45:10, by vol.)

In no case could an interfering chromogen be separated from a spot normally assayed. In addition, after completing "standard" runs of extracts, the separated spots were acetylated by spraying the plates with acetic anhydride: pyridine (50:50, v/v); after standing for 2 hr. they were then "run up" at 90° to the line of the first development,

thus forming new origins for a second development carried out using ethanol: benzene (2:98, v/v). For the compounds normally assayed no additional antimony trichloride staining spots could be separated.

Identification of Compounds. From extracts of infant urine, 50 to 100 μg. of AD, 16-O-AD, 16-OH-DHA, 16-OH-Preg and AT were prepared as follows: the extract was streaked along the origin of thin-layer chromatography plates which were then developed in the normal systems; the antimony trichloride reaction was carried out only on the two outer edges of the plates and the bands of material in the centre, thus delineated, were eluted from the silica-gel with ether, the silica-gel having first been deactivated with a drop of water. The compounds were converted to their trimethylsilyl ethers and subjected to gas-liquid chromatography using an E30L column. In each case they had the same retention times as the corresponding authentic standards treated in the same way.

The trimethylsilyl ether of each eluate was then subjected to gas chromatography-mass spectroscopy, and mass spectra were produced on the beginning, centre and end of each gas chromatography peak. In each case all three spectra were identical with each other and with similar spectra produced by the authentic standard steroids (the identification of Δ^5 steroids using gas chromatography-mass spectroscopy will be the subject to a later publication with Dr. C. J. W. Brooks).

Precision. The precision of the method (the concordance of measurements of the same quantity) has been determined by calculating the standard deviation from the differences between the chromatogram peak areas of duplicate extracts (duplicated through the whole procedure) of the

urinary assays reported in this paper. Table 4 shows the results obtained.

TABLE 4

Precision, as shown by the concordance of areas under the peaks of chromatogram scans.

Compound assayed	No. of duplicate assays	Average area (planimeter readings)	Standard deviation of the area (%)*
U_1	22	81	7.3
21-OH-Preg	22	43	8.9
AD	11	95	8.5
16-O-AD	22	252	4.5
16-OH-DHA	22	211	7.6
U_2	17	85	7.0
16-OH-Preg	22	245	4.4
AT	22	162	5.7

* The standard deviation has been calculated from the formula $\sqrt{\frac{\Sigma d^2}{2N}}$ where d = % difference between duplicate areas and N = no. of duplicate assays.

Labour involved. Working a normal 8 hr. day, it is possible for one reasonably skilled technician to assay 6 specimens each week.

Findings in normal individuals

Table 5 compares the excretion (in $\mu g/24$ hr/m^2) of the major identified Δ^5 steroids and two unknown Δ^5 steroid-like compounds (measured in terms of the chromogenicity of the Δ^5 steroid nearest in polarity, 21-OH-Preg and 16-O-AD) in the day-old infant with that found in normal adult males. AD, 16-O-AD, 16-OH-DHA, 16-OH-Preg and AT have

TABLE 5

URINARY EXCRETION OF Δ^5 STEROIDS

$\mu g/24\ hr/m^2$

STEROID	DAY OLD INFANT Average	DAY OLD INFANT Range	ADULT MALE Average	ADULT MALE Range
Dehydroepiandrosterone (DHA)	-	-	449	120 - 890 (10)
UNKNOWN 1	2,415	180 - 11,785 (11)	-	-
21-Hydroxypregnenolone (21-OH-Preg)	1,050	< 100 - 2,355 (11)	-	-
Androstenediol (17α + 17β) (AD)	860	< 100 - 4,200 (11)	370	73 - 417 (4)
16-oxoandrostenediol (16-O-AD)	4,515	435 - 12,100 (11)	99	<20 - 164 (4)
16-Hydroxydehydroepiandrosterone (16-OH-DHA)	4,810	300 - 11,200 (11)	745	201 - 871 (4)
UNKNOWN 2	3,330	470 - 7,600 (11)	-	-
16-Hydroxypregnenolone (16-OH-Preg)	4,135	260 - 7,600 (11)	-	-
Androstenetriol (AT)	3,200	420 - 8,100 (11)	341	200 - 555 (10)
Pregnenetriol	-	-	298	120 - 485 (10)
Total of averages	24,315		2,302	

- Denotes presence only in barely detectable or undetectable quantity.

The figures in parentheses give the number of subjects studied.

TABLE 6. URINARY EXCRETION OF Δ^5 STEROIDS IN EARLY INFANCY (μg/24 hr.)

AGE IN DAYS ( ) = no. of infants.

STEROID	1 (11)	2 (11)	3 (11)	4 (5)	5 (5)	6 (5)
	Average excretion with range					
UNKNOWN 1	483 36 - 2,357	555 285 - 1,608	616 374 - 1,221	683 320 - 1,690	429 212 - 895	450 150 - 675
21-Hydroxypregnenolone (21-OH-Preg)	210 15 - 471	236 40 - 492	279 128 - 590	311 104 - 690	193 42 - 435	165 28 - 320
Androstenediol (17α + 17β) (AD)	172 <20 - 840	191 <20 - 800	208 <20 - 880	144 <20 - 220	47 <20 - 120	37 <20 - 140
16-Oxoandrostenediol (16-O-AD)	903 87 - 2,420	1,060 80 - 3,000	1,502 285 - 3,260	1,518 660 - 2,080	1,031 680 - 1,680	899 340 - 1,480
16-Hydroxydehydroepiandrosterone (16-OH-DHA)	962 72 - 2,240	1,026 170 - 2,560	1,304 410 - 3,180	1,057 539 - 1,565	770 900 - 1,140	855 240 - 1,800
UNKNOWN 2	666 94 - 1,520	808 200 - 1,444	780 200 - 1,740	719 350 - 1,393	460 176 - 878	406 100 - 800
16-Hydroxypregnenolone (16-OH-Preg)	827 52 - 1,520	900 80 - 2,260	1,167 200 - 3,020	1,298 910 - 2,424	1,037 264 - 2,080	1,114 280 - 2,680
Androstenetriol (AT)	640 84 - 1,620	626 112 - 1,220	968 250 - 1,600	996 820 - 1,300	759 383 - 1,100	621 404 - 820
Total of averages	4,863	5,402	6,824	6,726	4,726	4,547

been identified by comparison with authentic standards during thin-layer chromatography in several systems and also by gas-liquid hromatography of their trimethylsilyl ethers followed by mass spectroscopy. DHA, 21-OH-Preg and pregnenetriol have only been partially identified from their Rf values in several thin-layer chromatography systems and the specificity of the antimony trichloride staining bands.

Table 6 shows the excretion during the first few days of life of the 8 compounds shown in Table 5 to be prominent in infant urine. There was a considerable variation in the excretion of each compound by separate individuals.

The excretion in 2 cases of the adrenogenital syndrome is shown in Table 7.

TABLE 7

URINARY EXCRETION OF Δ^5 STEROIDS IN TWO CASES OF THE ADRENOGENITAL SYNDROME

(μg/24 hr)

STEROID	CASE 1 aged 2 days	CASE 2 aged 7 days
UNKNOWN 1	150	1,350
21-Hydroxypregnenolone (21-OH-Preg)	not assayed	1,350
Androstenediol (17α and 17β) (AD)	150	638
16-oxoandrostenediol (16-O-AD)	100	6,750
16-Hydroxydehydroepiandrosterone (16-OH-DHA)	100	7,425
UNKNOWN 2	<20	995
16-Hydroxypregnenolone (16-OH-Preg)	1,550	14,925
Androstenetriol (AT)	200	1,460
Total	2,250	34,893

DISCUSSION

The relatively large number of Δ^5 steroids present in the biol gical material being assayed and the small quantity of original

Androstenetriol (AT)	84 - 1,620	4,863	112 - 1,220	5,402	250 - 1,600	6,824	820 - 1,300	6,726	383 - 1,100	4,726	404 - 820	4,547
Total of averages												

621 759 996 968 626 640

material available, led to the choice f a single technique which would maintain a reasonable degree of reproducibility while measuring all the compounds required, with a fairly high degree of specificity. The chromatograms produced also give a comprehensive indication of the amounts of other Δ^5 steroid-like compounds present, and this feature is important in view of the large number of these compounds and the present lack of knowledge as to their identity and importance.

All the steroids on the chromatograms which have been referred to by name have been identified before either in infant urine or fetal blood or tissue:
Preg (1,18), DHA (3,7), 21-OH-Preg (1,19), AD (1,2,9,20).
16-O-AD (9,21,22), 16-OH-DHA (2,5,7,8,9,23,24,25),
16-OH-Preg (1,2,5,8,9,24), AT (2,5,9,20,25,26). The bands labelled U_1 and U_2 together with the other unlabelled bands shown in Figs. 1 and 2 remain to be identified. In the present investigation only the last five compounds mentioned by name above (the major identified Δ^5 steroids in infant urine) have been fully characterized. AD includes both 17α and 17β androstenediol, separation of the epimers only being possible after further chromatography.

The precision obtainable is adequate for the purposes for which the technique is designed. The standard deviations shown in Table 4 are true for single assays, when in practice the average of duplicate measurements is used the precision is considerably increased.

The excretion of such a large quantity and variety of Δ^5 steroids in early life is undoubtedly related to the low activity of 3β-hydroxy steroid dehydrogenase which is known to obtain in the fetus and which must persist during early childhood. Similarly the high rate of

excretion f 16-OH-DHA in the absence of barely detectable amounts of DHA must be related to the known high activity of 16α-hydroxylase in the fetus and infant and not in the adult.

The results shown in Table 6 indicate that on the whole the compounds measured are being synthesized by the infant in fairly constant amount during the first 6 days of life. The normal newborn infant may be considered, because of its deficiency in 3β-hydroxy dehydrogenase, to possess a mild form of the congenital adrenal hyperplasia due to severe congenital deficiency of 3β-hydroxy dehydrogenase first described by Bongiovanni (27). In babies with this disorder, large quantities of Δ^5 steroids are produced (10, 27), presumably by overactivity of an adrenal under the stimulus of excessive corticotrophin from a pituitary endeavouring to maintain an adequate level of blood cortisol.

It is probable that the normal fetus and infant synthesize cortisol and corticosterone differently from the major route used by adults, the limited activity of 3β-hydroxy dehydrogenase acting subsequent to 17α, 21- and 11β-hydroxylase (9,10). This would account for the presence of 3β,21-dihydroxypregn-5-en-20-one in infant urine (19) and cord blood (1) and 3β,17α,21-trihydroxypregn-5-en-20-one and 3β,11β,17α, the C-19 Δ^5 steroids (DHA, 16-OH-DHA etc) would be incidentally increased in the same way that 17 oxosteroids are over-produced in the more common forms of congenital adrenal hyperplasia due to deficiency of 11β- and 21-hydroxylase. As with increasing age the deficiency of 3β-hydroxy dehydrogenase is rectified, cortisol production becomes more efficient and the "by-products" are no longer formed in quantity.

Of the compounds measured, AD, 16-O-AD, 16-OH-DHA and AT may be c nsidered t be estr gen precursors (r their metabolites) produced

by the fetus for aromatization in the placenta. 16-OH-Preg however is n t an immediate precursor of estrogen but is possibly derived, as is 16-OH-DHA, by 16 hydroxylation in the liver of the parent compound produced by the adrenal (20). The reported levels of pregnenolone in fetal blood vary considerably (23 μg/100 ml., (1); 45 μg/100 ml., (18); 200 μg/100 ml., (28)). It can be utilized by the placenta for progesterone production (29) and by the fetal adrenal for DHA production (30). Further work is required to elucidate the reason for its considerable quantitative significance as indicated by the large amount of 16-OH-Preg excreted in infant urine.

In 2 cases of the adrenogenital syndrome whose results are shown in Table 7, there is a selective increase in the excretion of 16-OH-Preg. Case 1 shows a reduction in the excretion of the other compounds measured, 21-tetrahydroxypregn-5-en-20-one in cord blood (1). If, in the normal infant, excess corticotrophin is being produced to stimulate normal cortisol production in the face of a lack of 3β-hydroxy dehydrogenase, but Case 2 shows a considerable increase in all compounds. Reynolds (31) has measured 16-OH-Preg and 16-OH-DHA excretion in cases with 21-hydroxylase deficiency and has shown that during the first few months of life the ratio of 16-OH-Preg/16-OH-DHA is increased from a normal average of 3:2 to 10:1 in the adrenogenital group. As for normal infants he found that both steroids were almost undetectable in the urine of patients aged over 5 months. The marked increase in 16-OH-Preg excretion in the cases reported here, together with the findings of Reynolds would seem to indicate that in the patients studied there was for some reason a relative inactivity of 17-20 desmolase. It is

difficult to suggest a reason for this and further study is required. The considerable difference from normal in the excretion of Δ^5 steroids by cases of the adrenogenital syndrome indicates the importance of their assay in the investigation of such disorders.

Work carried out in recent years has uncovered the existence of complex interrelationships in the steroid metabolism of the mother, the fetus and the placenta (9), but much of this work has so far only been qualitative. The Δ^5 steroids certainly play a major role and the use of the technique described, should expedite work on the quantitative relationships of these steroids in the feto-placental unit and in the newborn, and facilitate the isolation and identification of more compounds. As thin-layer chromatography forms the basis of the separations, the chances of transformation, destruction or artifact formation taking place are considerably less than would be likely with the alternative gas-liquid chromatography.

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The authors wish to thank Dr. P. J. Sykes and Mr. R. W. Kelly for synthesizing many steroids, Dr. C. J. W. Brooks for helpful collaboration with gas chromatography-mass spectroscopy, Professor W. Klyne and the Medical Research Council Steroid Reference Collection for many samples of steroids, Dr. D. Fukushima for a supply of 16-OH-DHA, Dr. J. Livingstone and Mrs. E. A. Michie for collecting the specimens of amniotic fluid and cord blood, Sisters M. Barr and C. Wilson for baby urine collections, Dr. J. K. Grant for supplying urine from a case of adrenogenital syndrome, Miss C. A. Chitty and Miss E. J. Lawrie for technical assistance and the Medical Research Council for financial assistance.

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STEROID EXCRETION AND BIOSYNTHESIS WITH SPECIAL REFERENCE TO C_{19}-Δ^{16}-STEROIDS IN AN INFANT WITH A VIRILIZING ADRENOCORTICAL CARCINOMA

By

D. B. Gower, J. R. Daly, G. J. A. I. Snodgrass* and Margaret I. Stern***

ABSTRACT

A case of a 15-month old girl with a virilizing adrenocortical carcinoma is described. Three C_{19}-Δ^{16}-steroids, 3α-hydroxy-5α-androst-16-ene, 3α-hydroxy-5β-androst-16-ene and 3β-hydroxy-androsta-5,16-diene, have been obtained from the glucuronide fraction of the urine and identified by column and gas-liquid chromatographic techniques. Estimation of these compounds, not normally present in infants' urine, showed that adult female amounts were being excreted, although post-operatively very little was detected. Androsterone, aetiocholanolone and especially dehydro*epi*androsterone were present in excessive amounts preoperatively; approximately one-third of the dehydro*epi*androsterone was excreted as glucuronide, the rest as sulphate. The adrenal tumour metabolized [4-^{14}C] pregnenolone and [4-^{14}C] progesterone to 4.7 % and 0.15 % respectively of 3β-hydroxy-androsta-5,16-diene. The evidence for biosynthesis of this compound from progesterone supports that obtained by other workers for the production of Δ^5- from Δ^4-steroids. Labelled androstenedione was also obtained from both substrates and 11β-hydroxyandrostenedione from progesterone. Only small quantities of radioactivity were recovered in the glucuronide and sulphate fractions from the tissue incubations.

Functioning tumours of the adrenal cortex are rare in childhood. Over the last hundred years only 250 cases have been reported since the original patient of

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D BIOSYNTHESIS WITH
^{16}-STEROIDS IN AN INFANT
NOCORTICAL CARCINOMA

*, G. J. A. I. Snodgrass**
t I. Stern***

RACT

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Pitman (1865) and *Ogle* (1865). The experience of even large centres has been limited to only a few cases. Four examples were found out of 82 220 in-patients in one hospital over 26 years by *Garrett* (1951). Twelve further cases occurred in the Mayo Clinic over a 30 year period (*Hayles et al.* 1966). This group also reviewed 222 other cases from the literature and found that the tumours were usually malignant and that virilization was the commonest clinical presentation. Recently, the case of a girl of 15 months, showing signs of masculinization due to a hormone producing tumour of the right adrenal gland has been described (*Sekouri & Dardougia* 1968). The tumour was removed *in toto* and histological examination revealed an adrenal carcinoma. The child's subsequent course was uneventful. No biosynthetic studies were carried out on the tumour, but urinary 17-oxosteroid and 17-hydroxycorticosteroid values were similar to the results presented here, both pre- and post-operatively.

Except for the more recently reported cases, biochemical data has been scanty. In cases of carcinoma where virilization is pronounced and the urinary excretion of 17-oxosteroids high, the main steroid metabolite recovered in the urine is usually DHA*. A considerable number of steroids has been detected in the urine of such cases, and the present report concerns an infant with an adrenocortical carcinoma who excreted C_{19}-Δ^{16}-steroids. Although 3α-androstenol is a normal constituent of the urine of both adult males and females (*Brooksbank & Haslewood* 1961), it has not previously been detected in the urine of infants (*Gower*, unpublished) nor of pre-pubertal children (*Cleveland & Savard* 1964). 3α-androstenol excretion may be increased in cases of adrenal tumour (*Burstein & Dorfman* 1962; *Gower & Stern* 1969), adrenal hyperplasia (*Mason & Schneider* 1950), and luteoma of the ovary (*Engel et al.* 1953). 3α-androstenol was detected in the urine of our patient together with small quantities of two other C_{19}-Δ^{16}-steroids, aetiocholenol and androstadienol, also not previously detected in the urine of a child. *Burstein & Dorfman* (1962) have shown that cholesterol and pregnenolone are precursors of 3α-androstenol *in vivo* in a woman with virilizing adrenal adenoma. *Gower & Stern* (1969) found that pregnenolone was metabolized to androstadienol, and progesterone to androsta-4,16-dien-3-one by adrenal carcinoma minces. It was of interest, therefore, to measure C_{19}-Δ^{16}-steroid excretion in the present case and to study the formation of this group of steroids by the diseased adrenal tissue.

Of additional interest was the hypertension in our patient which was relieved following removal of the tumour. 1-Oxygenated steroids were detected

* Abbreviations used are as follows: 3β-androstenol, 3β-hydroxy-5α-androst-16-ene; 3α-androstenol, 3α-hydroxy-5α-androst-16-ene; aetiocholenol, 3α-hydroxy-5β-androst-16-ene; androstadienol, 3β-hydroxy-androsta-5,16-diene; DHA, dehydro*epi*-androsterone.

in the patient's urine pre-operatively, but were not present in the post-operative samples. These compounds have been detected previously in human urine on two occasions; both patients were infants with hypertension (*Edwards et al.* 1968). The details of the 1-oxygenated steroid excretion in the urine of this patient will be published elsewhere.

CASE HISTORY

The patient, a girl, was delivered normally at home at 36 weeks gestation. The birth weight was 2500 g. She was the second child of young parents. The elder sibling, also a girl, is completely normal. The pregnancy had been complicated by a threatened abortion at 8 weeks gestation but no hormonal preparations were given at any time.

The parents thought the clitoris was unduly prominent at birth but the attending personnel did not comment on this. No other abnormalities were noted at this time.

Although the child thrived, her mother noticed that the clitoridal enlargement had increased relative to her general growth by the age of 6 months. She did not seek medical aid then nor even when pubic hair began to appear at the age of 8 months. Under pressure from her relatives, the mother sought paediatric advice when the patient was aged one year.

On admission she was found to be a muscular child of a strikingly outgoing temperament. Mental development was normal for her age. A discrete acneiform rash was present over the forehead and hirsuties were noted over the paravertebral region as well as the limbs. Surprisingly, considering the evidence of virilism, her supine length was only 73.6 cm (50th percentile) but she weighed 11.2 kg (90th percentile). The head circumference was normal at 47.9 cm.

Detailed examination of the genitalia revealed scanty, coarse and curly pubic hair of a feminine distribution. The clitoris was 2 cm long. The labia majora and minora were enlarged, the vaginal and urethral orifices normally situated. Rectal examination confirmed the presence of a uterus.

The only other abnormality noted in any system was a persistently elevated blood pressure. At the initial examination this was 160/85 mmHg but had increased to 180/110 mmHg prior to operation.

A buccal smear was chromatin positive and 3% of the neutrophils showed drumstick formation.

Operation

The abdomen was explored via a right upper para-median incision. The left adrenal was first exposed and found to be normal in size and appearance.

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No recognisable adrenal tissue was seen on the right side. Instead there was a well encapsulated tumour the size of a hen's egg. This was removed and found to weigh 50 g. No invasion of the para-renal tissues was seen, nor extension into the local vasculature. Peripheral venous and right adrenal vein blood was obtained before removal of the tumour. Microscopical examination of the tumour (Professor J. C. Sloper) showed it to have a thin fibrous capsule and to be composed of sheets and trabeculae of large cells mostly eosinophilic and granular. There was no suggestion of zonal arrangement. Considerable nuclear pleomorphism was seen and also scattered areas of calcification suggesting the tumour had been present for some time. The diagnosis was that of adrenocortical carcinoma.

Post-operative management

The patient was maintained on intravenous fluids and gastric suction for 5 days following operation. Cortisol 60 mg was given by this route over the first 24 h and 30 mg and 15 mg over the following 2 days. Simultaneously, ACTH 60 units was given daily by continuous infusion for a total of 5 days.

Recovery was rapid thereafter and she was discharged home three weeks later, well and receiving no medication. She has remained well since that time (2 years).

EXPERIMENTAL

Materials and Methods

Purification of solvents, preparation of alumina and Kieselgel G used for chromatography have been described earlier (*Gower & Ahmad* 1967).

For the investigation and analysis of Δ^{16}-steroids, androsterone, aetiocholanolone and DHA in urine, samples were processed as described earlier (*Gower & Stern* 1969) and hydrolysed extracts chromatographed on columns of alumina (*Brooksbank & Gower* 1970). Two fractions were obtained using 41–90 ml and 91–150 ml of a mixture of light petroleum (80–100°C):benzene (1:1, v/v). The first of these normally contains 3α-androstenol and the second aetiocholenol and androstadienol (*Brooksbank & Gower* 1970). Gas-liquid chromatography (GLC) of the chloromethyldimethylsilyl (CMDS) ethers of Δ^{16}-steroid fractions was performed as described by *Brooksbank & Gower* (1970). For further identification the bromomethyldimethylsilyl (BMDS) ethers were also prepared using a modification of the method of *Thomas & Walton* (1968). Following these non-polar fractions, androsterone, aetiocholanolone and DHA were eluted together from alumina using 75 ml of benzene containing 0.5 % of ethanol and were estimated by GLC of their trimethylsilyl ethers (*Kirschner & Lipsett* 1963).

Other methods used for the estimation of urinary steroids are given in Table 1.

Plasma testosterone was measured by the method of *Collins et al.* (1968).

In vitro studies

1 g portions of minced adrenal tumour tissues were incubated for three hours in

5 ml Krebs bicarbonate buffer, pH 7.3–7.4 with 1.5 μCi each of [4-^{14}C] pregnenolone (specific activity 24 mCi/mM) and [4-^{14}C] progesterone (specific activity 58.5 mCi/mM) and co-factors as described earlier (*Gower & Stern* 1969). After incubation and extraction with 3 × 5 ml ethyl acetate, 50 μg of each of the following carrier steroids was added: 3α- and 3β-androstenols, aetiocholenol, androstadienol, 3-hydroxy-oestra-1,3,5(10),16-tetraene, androsta-4,16-dien-3-one, androstenedione, DHA, progesterone, pregnenolone and testosterone. The pooled ethyl acetate extract was evaporated to dryness under reduced pressure, on a warm water bath. The pH of the remaining aqueous mixture was adjusted to approximately 2.0 with concentrated HCl, ammonium sulphate (1:2, w/v) was added, and extraction carried out three times with ether: ethanol (3:1, v/v) (*Edwards et al.* 1953). The organic solvent was evaporated to dryness, 15 ml of 0.1 M acetate buffer (pH 4.5) and 2 ml Ketodase (William R. Warner & Co. Ltd., Eastleigh, Hants., England) added to each flask and the mixtures were incubated at 37°C for 48 h. After adjusting the pH to approximately 1.0, the aqueous mixture was extracted three times with 10 ml ether, and the ether evaporated. This extract was designated the G (Glucuronide) fraction. The remaining aqueous portion was solvolysed according to *Magendantz & Ryan* (1964). The resulting ethyl acetate was evaporated to dryness and designated the S (solvolysed) fraction. The initial ethyl acetate extracts and the G and S fractions were chromatographed on alumina, essentially as described earlier (*Gower & Ahmad* 1967) unless the radioactivity was too small for further studies to be performed.

Separation and tentative identification of radioactive metabolites was achieved by submitting each alumina column fraction to thin layer chromatography followed by radioautography and preparation of derivatives, where possible, as described in detail earlier (*Gower* 1966; *Gower & Stern* 1969). It was possible to characterise one Δ^{16}-steroid metabolite by column chromatography (see Results section).

RESULTS

Urine

Results obtained from analysis of urine are given in Table 1. Total 17-oxosteroids were markedly raised and gradient elution chromatography showed the presence of a considerable quantity of DHA. GLC revealed that one-third of this quantity was excreted as glucuronide. These values had returned to normal one year after operation. Oestrogen excretion was small in amount and pre- and post-operative values did not differ markedly. Pregnanetriol was found in each urine specimen examined, still being present a year after operation, at which time pregnanediol was only just detectable. Pre-operatively, urinary testosterone gave a value in the normal adult female range but decreased post-operatively. Pregnenetriol before operation was only just detectable.

Investigation of C_{19}-Δ^{16}-steroids

Figs. 1 and 2 show the GLC tracings obtained by chlorosilanisation of the »Δ^{16}-steroid« fractions obtained from alumina. The »androstenol« fraction contained a compound with a retention time (RRT) similar to that of the

Table 1.
Urinary steroid levels (mg or mg/24 h) in an infant with a virilising adrenal carcinoma*

i–7.4 with 1.5 μCi each of [4-^{14}C] pregnenolone
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50 μg of each of the following carrier steroids
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Steroid		Pre-operative	Post-operative, within 2/52	Post-operative one year	Normal values for infants	Method
Total 17-OS		13.5–28.0	0.3–0.6	1.6–2.4	1.4 ± 0.7	*Drekter et al.* (1952) with modified Zimmermann
DHA	G	7.5	–	6*	0.1	*Brooksbank & Gower* (1970)
	S	14.1	–			
Androsterone	G	1.3	–	25*	0.1	*Kirschner & Lipsett* (1963) modified
	S	1.3	–			
Aetiocholanolone	G	1.4	–	50*	0.1	
	S	0.2	–			
3α-Androstenol		0.51	–	14*		
Aetiocholenol		6*	–	N. D.	N. D.	
Androstadienol		25*	–	N. D.		
Testosterone		10.0*	6.4*	<5.0*	<5.0*	*Brooks* (1964) modified
*Epi*testosterone		<5.0*	<5.0*	<5.0*	<5.0*	
Total 17-OHCS		1.5–3.5	0.3–0.6	1.6–2.4	<2	*Few* (1961) modified *James & Caie* (1964)
Pregnanetriol		0.3	1.1	0.5	<0.1	*Stern* (1957)
Pregn-5-en-3β, 17α,20α-triol		<0.1	–	–	<0.1	*Stern & Barwell* (1963)
Pregnanediol		1.6	0.3	<0.1	<0.1	*Klopper et al.* (1955)
Oestradiol		<1.0*	<1.0*	–	<0.1*	*Brown* (1955) modified by *Brown et al.* (1957)
Oestrone		2.2*	<1.0*, 2.6*	–	<0.1*	
Oestriol		1.1*	1.2*, 1.1*	–	<0.1*	

N. D. = not detected
G = Glucuronide
– = not measured
S = Sulphate

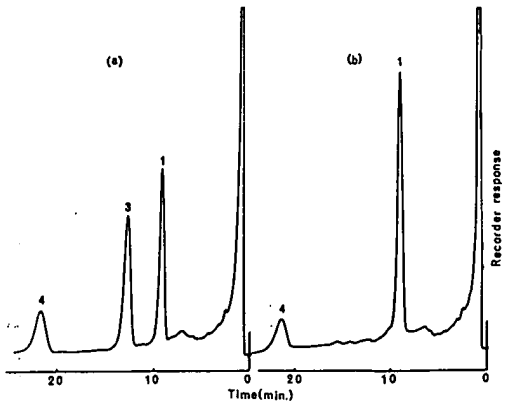


Fig. 1.

(a) GLC of a mixture of the CMDS ethers of 3α-androstenol (peak 1) and androstadienol (peak 3);
(b) »3α-androstenol« fraction (eluted from alumina and chlorosilanised) obtained after hydrolysis of urinary glucuronides of an infant with a virilising adrenocortical carcinoma. Cholestane (peak 4) was added as internal standard. Column: CDMS/JXR (0.6 %/0.75 %) and conditions as Table 2.

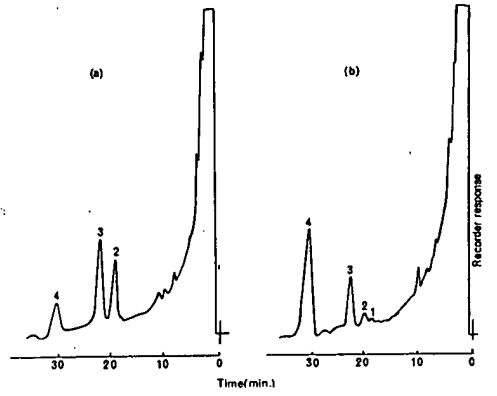
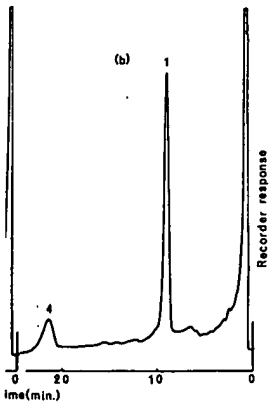


Fig. 2.

(a) GLC of the CMDS ethers of aetiocholenol (peak 2) and androstadienol (peak 3);
(b) »androstadienol« fraction (eluted from alumina and chlorosilanised) obtained as in Fig. 1. Cholestane (peak 4) was added as internal standard. Column: QF_1 (5 %) and conditions as Table 2.

CMDS ether of authentic 3α-androstenol, relative to the internal standard, cholestane (Fig. 1). The second alumina column fraction contained compounds which had RRTs corresponding to the CMDS ethers of aetiocholenol and androstadienol. A trace of the CMDS ether of 3α-androstenol, not separated



ig. 1.
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as internal standard. Column: CDMS/JXR
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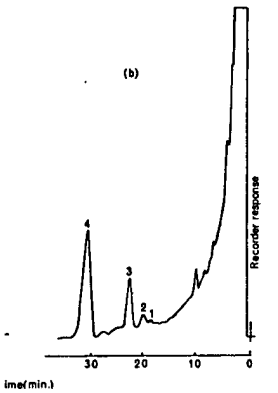

ig. 2.
olenol (peak 2) and androstadienol (peak 3);
m alumina and chlorosilanised) obtained as
ed as internal standard. Column: QF_1 (5 %)

tenol, relative to the internal standard,
na column fraction contained compounds
the CMDS ethers of aetiocholenol and
S ether of 3α-androstenol, not separated

on alumina, was also present in this fraction (Fig. 2). Proof of identity was obtained by GLC on two columns of the BMDS ethers prepared from further portions of the alumina column fractions. In each case the RRTs were identical to those of the authentic derivatives (Table 2). The data also show that 3β-androstenol was absent in this urine, a finding in keeping with the earlier work of *Brooksbank & Gower* (1964).

Estimation of Δ^{16}-steroids in the pre-operative urine revealed that the excretion of 3α-androstenol (0.51 mg/24 h) was equivalent to that of an adult woman. In normal infants, the urinary 3α-androstenol is known to be either very low or undetectable (*Gower*, unpublished), and in the present case the values of urinary 3α-androstenol post-operatively were very low (Table 1). The levels of aetiocholenol and androstadienol pre-operatively were 6 and 25 μg/24 h which are approximately those found in adult women (*Brooksbank & Gower* 1970). These compounds have not so far been detected in the urine of normal infants (*Gower*, unpublished).

Table 2.
GLC of Δ^{16}-steroids obtained from urinary glucuronide fraction of a child with virilizing adrenal carcinoma. Chloromethyl dimethyl silyl (CMDS) ethers were run at 190°C and bromomethyl dimethyl silyl (BMDS) ethers at 200°C..

	Retention times relative to cholestane (= 1)			
	CDMS/JXR*		QF_1**	
	Authentic steroid	Urine	Authentic steroid	Urine
CMDS ethers				
3α-Androstenol	0.415	0.417	0.606	0.604
Aetiocholenol	0.488	0.484	0.610	0.613
Androstadienol	0.580	0.582	0.730	0.731
3β-Androstenol	0.580	0.582	0.795	–
BMDS ethers				
3α-Androstenol	0.580	0.583	0.750	0.744
Aetiocholenol	0.677	0.670	0.786	0.780
Androstadienol	0.840	0.830	0.910	0.916
3β-Androstenol	0.840	0.830	1.16	–

Columns used: Cyclohexane dimethanol succinate (CDMS)/methyl polysiloxane (JXR) (0.6 %/0.75 %) and fluoralkyl silicone, QF_1 (5 %) with carrier gas 50 ml/min.
Cholestane times: * 32.5 and 29.8 min at 190°C and 200°C respectively.
** 35.9 and 33.0 min at 190°C and 200°C respectively.

Plasma

Pre-operatively the level of peripheral plasma testosterone was 142 ng/100 ml and the adrenal venous plasma concentration was 263 ng/100 ml.

Biosynthetic studies

The amounts of radioactivity extracted from the adrenal tissue incubations with ethyl acetate and obtained after hydrolysis and solvolysis are shown in Table 3. Virtually all the radioactivity was extracted with ethyl acetate and there was no residual radioactivity in the aqueous layers after hydrolysis and extraction of conjugates. In view of the low content of radioactivity of the conjugate fractions, they were not investigated further. The ethyl acetate-extractable material, however, was chromatographed on alumina columns as described earlier (*Gower & Ahmad* 1967). Only 66.5 % and 22.9 % respectively of the radioactivity from the pregnenolone and progesterone incubations was eluted using solvents with increasing polarity up to benzene containing ethanol (1 %) (see *Gower & Ahmad* 1967). Elution with ethanol (50 ml) resulted in the recovery of a further 6–10 % of radioactivity. Only when 50 ml of aqueous methanol (1:1, v/v) was used was the remaining radioactivity recovered (Table 4). Over 20 % was obtained from the pregnenolone and as much as 58 % from the progesterone incubations respectively. This has been noticed repeatedly using adrenal tissue especially with radioactive progesterone as precursor.

Biosynthesis of Δ^{16}-steroids

Fractions which might contain C_{19}-Δ^{16}-steroids were obtained by alumina column chromatography of the ethyl acetate extracts using 41–150 ml of a benzene-light petroleum mixture (1:1, v/v) (*Gower & Ahmad* 1967). These

Table 3.

Percentage of radioactivity obtained by extraction and hydrolysis of steroid conjugates after incubation of adrenal carcinoma with [4-^{14}C] pregnenolone and [4-^{14}C] progesterone.

Precursor	Ethyl acetate-extractable	Glucuronides	Sulphates	Aqueous layer
Pregnenolone	98.3	1.4	0.21	nil
Progesterone	98.8	1.0	0.07	nil

Adrenal carcinoma minces were incubated with [4-^{14}C] pregnenolone and [4-^{14}C]-progesterone and extracted with ethyl acetate (3 × 5 ml). Conjugates remaining were extracted, hydrolysed and solvolysed separately and extracted with ethyl acetate (3 × 5 ml).

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^{14}C] progesterone.

Glucuronides	Sulphates	Aqueous layer
1.4	0.21	nil
1.0	0.07	nil

ɔated with [4-^{14}C] pregnenolone and [4-^{14}C]-
yl acetate (3 × 5 ml). Conjugates remaining
'olysed separately and extracted with ethyl

Table 4.
Elution of ethyl acetate-extractable radioactivity (obtained as in Table 3) from alumina columns.

Substrate	Fractions I–VI of *Gower & Ahmad* (1967)	Ethanol (50 ml)	Aqueous methanol (1:1, v/v, 50 ml)	Total
[4-^{14}C] pregnenolone	66.5	6.2	20.8	93.5
[4-^{14}C] progesterone	22.9	10.3	58.2	91.4

Values given are percentages of radioactivity added to the columns.

were evaporated to small bulk and portions removed for measurement of radioactivity. The extract from the pregnenolone incubation contained approximately nine times as much radioactivity as that from the progesterone incubation. The remaining portions were chromatographed on thin layers of Kieselgel G using benzene-ether (9:1, v/v) as solvent (*Gower* 1964). Radioautography revealed radioactive zones in both extracts which ran with the mobility of authentic androstadienol (Fig. 3 a). Other minor radioactive zones have not been identified. The two major zones were investigated further by running them on thin layers of silver nitrate-impregnated silicic acid, using benzene-ethyl acetate (1:1, v/v) as solvent since, under these conditions, androstadienol can be resolved easily from 3β-androstenol (*Lisboa & Palmer* 1967). Radioautography revealed that in both cases the radioactivity was associated only with the marker androstadienol (Fig. 3 b). In order to confirm the identity of this metabolite it was eluted from the TLC plate and submitted to chromatography on a column containing 3 g alumina (partially deactivated with 4.5 % water (*Gower & Ahmad* 1967)) and 3 g of Kieselgel H which had previously been impregnated with silver nitrate (0.66 g). Using a mixture of authentic androstadienol and 3β-androstenol, it was known (*Katkov*, unpublished) that the latter could be eluted using approximately 60 ml of a mixture of benzene-ethyl acetate (2:1, v/v). Androstadienol was then eluted with approximately 60 ml of benzene-ethyl acetate – ethanol (40:20:1, by volume). Further details of this separation will be published elsewhere*. After an initial bulk fraction (30 ml), 2 ml fractions were collected until all radioactivity had been eluted. In each fraction the radioactivity was measured by liquid scintillation counting and the weight of added carrier steroid by GLC (*Gower & Thomas* 1968). Figs. 4 and 5 show that there was no radioactivity associated with 3β-androstenol, and

* *Katkov T. & Fower D. B.: Biochem. J.* (in press).

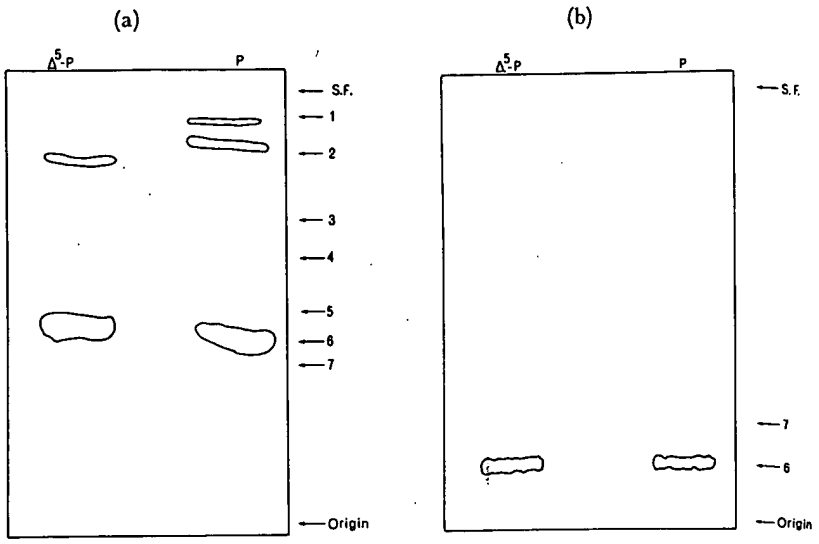


Fig. 3.

(a) Radioautograph (tracing) of radioactive Δ^{16}-steroid fractions (eluted from alumina) obtained from incubation of [4-^{14}C] pregnenolone (Δ^5-P) and [4-^{14}C] progesterone (P) with virilising adrenocortical carcinoma tissue. Extracts were run on Kieselgel G three times in benzene-ether (9:1, v/v).

(b) Radioautograph (tracing) of the »androstadienol« zones after TLC as in Fig. 3 (a) run on Kieselgel G impregnated with silver nitrate. Solvent was benzene-ethyl acetate (1:1, v/v). Marker steroids: 1, 5α-androst-16-en-3-one; 2, 3-hydroxy-oestra-1,3,5(10),16-tetraene; 3, androsta-4,16-dien-3-one; 4, 3α-androstenol; 5, aetiocholenol; 6, androstadienol; 7, 3β-androstenol. S. F. = solvent front.

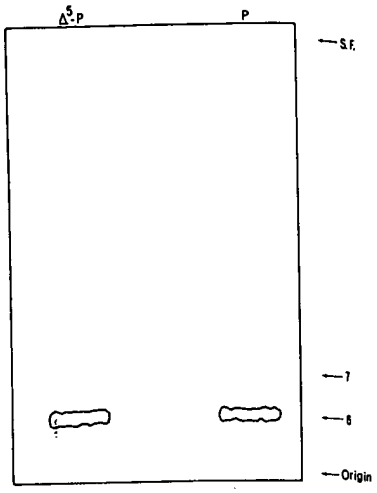
that radioactivity and mass peaks for androstadienol coincide. The specific activity of androstadienol (counts/min/μg) was constant over the peak in each case (Table 5) indicating that radiochemical purity had been achieved. Fig. 5 also shows that there was a small peak of radioactivity eluted from the column just after the carrier 3β-androstenol. It is possible that this may be due to androsta-4,16-dien-3-one formed by oxidation of androstadienol. The ketone is known to be incompletely separated from 3β-androstenol on this column.

Analytical losses were calculated by expressing the total weight of carrier androstadienol eluted from the column as a percentage of the amount originally added to the tissue incubation. It was then found that the adrenocortical tissue had metabolized radioactive pregnenolone and progesterone to androstadienol in yields of 4.7 % and 0.15 % respectively.

Biosynthesis of other steroids

(a) *From [4-^{14}C]pregnenolone.* By running the extracts in three different solvent systems on TLC, formation of derivatives and comparison with the

(b)



. 3.

Δ^{16}-steroid fractions (eluted from alumina) nolone (Δ^5-P) and [4-^{14}C] progesterone (P) sue. Extracts were run on Kieselgel G three

stadienol« zones after TLC as in Fig. 3 (a) r nitrate. Solvent was benzene-ethyl acetate i-en-3-one; 2, 3-hydroxy-oestra-1,3,5(10),16- α-androstenol; 5, aetiocholenol; 6, androsta- front.

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running the extracts in three different of derivatives and comparison with the

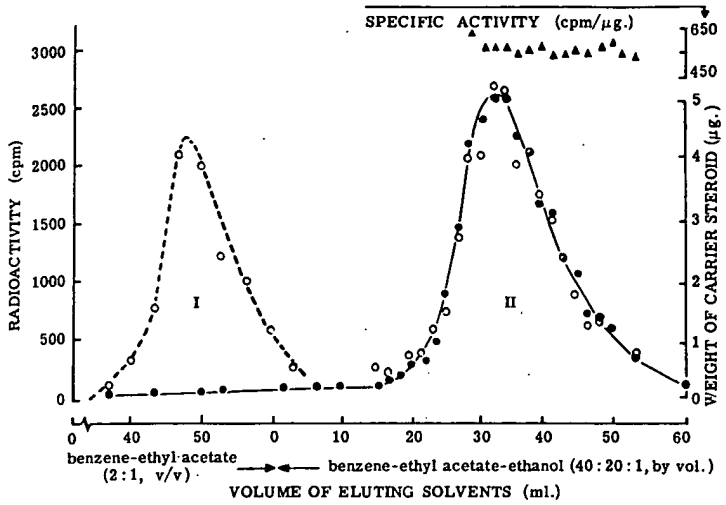

Fig. 4.

Purification of radioactive androstadienol formed by incubating [4-^{14}C] pregnenolone with adrenocortical carcinoma tissue. Radioactive androstadienol was isolated by TLC (Figs. 3 a and b), carrier 3β-androstenol (I) and androstadienol (II) added, and the mixture eluted from a column of Kieselgel H impregnated with silver nitrate (see text). The weight (o) of carrier steroids was measured by GLC and radioactivity (●) by liquid scintillation counting.

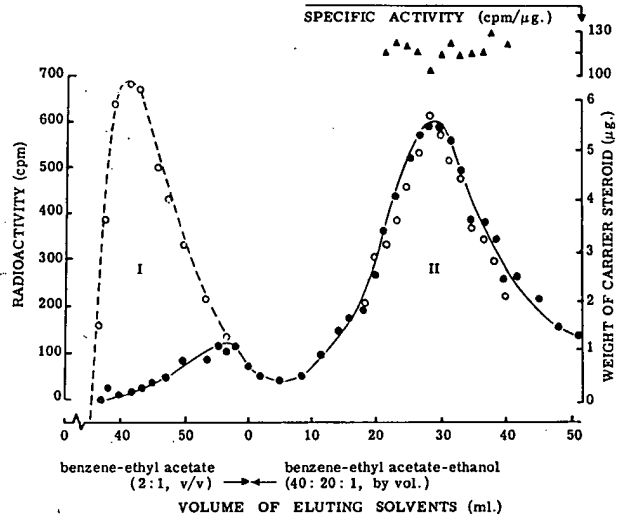

Fig. 5.

Purification of radioactive androstadienol formed by incubating [4-^{14}C] progesterone with adrenocortical carcinoma tissue. Details as legend to Fig. 4.

Table 5.
Specific activity of androstadienol obtained biosynthetically and purified as in Figs. 4 and 5.

Data from	Number of estimations	Mean (counts/min/μg)	Range	SD	Coefficient of variation (%)
Fig. 4	15	560.3	525–650	34.5	6.1
Fig. 5	12	117.7	106–128	5.8	4.9

relevant authentic steroids, tentative evidence for the formation of androstenedione was obtained, and for the presence of unchanged pregnenolone. There were several other compounds in small quantities. No evidence was obtained for the presence of testosterone, progesterone, DHA, or 11β-hydroxyandrostenedione in detectable quantities.

(b) *From [4-^{14}C]progesterone.* Tentative evidence was obtained for the presence of androstenedione and unchanged progesterone as well as 11β-hydroxyandrostenedione, by formation of derivatives and running in three different TLC systems with the relevant authentic steroid. No testosterone could be detected in the extract after incubation.

DISCUSSION

To our knowledge, this is the first case of an infant with an adrenal carcinoma in which Δ^{16}-steroid excretion and biosynthesis have been investigated. Previous studies have shown that in two virilized women who had an adrenal adenoma (*Burstein & Dorfman* 1962) or carcinoma (*Gower & Stern* 1969), abnormally high amounts of 3α-androstenol were excreted in the urine. The urine of our patient contained adult female amounts of 3α-androstenol and two other Δ^{16}-steroids (aetiocholenol and androstadienol), not usually detectable in infant urine (*Gower,* unpublished).

Moreover, evidence was obtained to show that the diseased adrenal could produce *in vitro* relatively large yields of androstadienol from pregnenolone. These results are in agreement with those obtained from a former patient although the yield of androstadienol was only 0.2 % (*Gower & Stern* 1969) and from recent *in vivo* experiments (*Brooksbank & Wilson* 1969).

The formation of a small amount of radioactive androstadienol from progesterone is unexpected, and if this is correct it is difficult to interpret. This observation would be explicable if progesterone were converted to pregnenolone, by a reversal of the Δ^{5}-3β-hydroxysteroid dehydrogenase-isomerase

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Figs. 4 and 5.

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CUSSION

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biosynthesis have been investigated. Pre-
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62) or carcinoma (*Gower & Stern* 1969),
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lt female amounts of 3α-androstenol and
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t of radioactive androstadienol from pro-
is correct it is difficult to interpret. This
progesterone were converted to pregneno-
3-hydroxysteroid dehydrogenase-isomerase

system. In recent years, evidence has accumulated to suggest that this can occur (*Ward & Engel* 1964, 1966*a,b*; *Rosner et al.* 1965).

It is noticeable that, despite the urinary excretion of DHA, this compound could not be detected in biosynthetic studies after incubation with [4-^{14}C]-pregnenolone.

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The authors are grateful to Dr. Hugh Jolly and Mr. Peter Philip who had clinical charge of the patient. Dr. D. B. Gower thanks Mr. D. C. Bicknell for valuable assistance and the Medical Research Council (Grant no. G. 967/305/B) and Guy's Hospital Endowments Fund for financial support. Dr. M. I. Stern thanks members of the Endocrine Unit who carried out some of the analyses.

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THE IDENTIFICATION OF C_{19}-16-UNSATURATED STEROIDS AND ESTIMATION OF 17-OXOSTEROIDS IN BOAR SPERMATIC VEIN PLASMA AND URINE

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WITH AN APPENDIX BY D. B. GOWER AND R. L. S. PATTERSON

(*Received* 20 *January* 1970)

SUMMARY

C_{19}-16-unsaturated steroids have been extracted from the urine and spermatic vein plasma of a mature boar and identified by thin-layer chromatography, gas–liquid chromatography and combined gas–liquid chromatography–mass spectrometry. 5α-Androst-16-en-3β-ol (approximately 250 μg./l.) was identified in the urinary glucuronide fraction. This compound and the 3α-epimer occurred in the spermatic vein plasma predominantly as sulphates but a small quantity of the 3α-isomer was extractable with ether prior to hydrolysis of steroid conjugates. Traces of 5,16-androstadien-3β-ol have been tentatively identified in the plasma sulphate fraction; 5α-androst-16-en-3-one occurred as free steroid. No 16-unsaturated steroids were found in the plasma glucuronide nor in urinary sulphate fractions. The latter contained an unidentified compound of similar polarity to the C_{19}-16-unsaturated steroids.

Neutral 17-oxosteroids were measured in extracts obtained from both the urine and spermatic vein plasma. Of the dehydroepiandrosterone (DHA) in the urine 60% occurred as sulphate and 40% as glucuronide with only traces as free steroid. Androsterone and aetiocholanolone occurred only as glucuronides. In the spermatic vein plasma, DHA occurred predominantly as sulphate with small amounts as glucuronide and free steroid.

INTRODUCTION

A quarter of a century ago two C_{19}-16-unsaturated steroids were isolated from boar testes by Prelog & Ruzicka (1944). In keeping with these early results, the biosynthesis of these and other 16-unsaturated steroids in testicular tissue has been well documented recently (Gower & Ahmad, 1967; Ahmad & Gower, 1968; Katkov & Gower, 1968). 16-Unsaturated steroids have been found elsewhere in the boar and Patterson (1968*a*, *b*) has obtained evidence for the presence of 5α-androstenone in the fat and saliva of mature animals. The submaxillary glands contain 3α-androstenol (Patterson,

1968*b*) and can metabolize pregnenolone to androstadienol *in vitro* (Gower & Katkov, 1969*a*).

Although the presence of 16-unsaturated steroids in human urine and plasma has been described (e.g. Brooksbank & Haslewood, 1961; Brooksbank & Gower, 1970; Brooksbank, Cunningham & Wilson, 1969; Gower & Stern, 1969), boar urine and plasma have not been investigated. For this reason and in view of the possible physiological significance of 16-unsaturated steroids in the boar (Melrose, Patterson & Reed, 1968), urine and spermatic vein blood from a boar were analysed. Some of these results have been described briefly elsewhere (Gower & Katkov, 1969*b*; Gower, Harrison, Heap & Patterson, 1970).

The following abbreviations are used: 5α-androstenone, 5α-androst-16-en-3-one; androstadienone, 4,16-androstadien-3-one; 3α- and 3β-androstenols, 5α-androst-16-en-3α- and -3β-ol; aetiocholenol, 5β-androst-16-en-3α-ol; androstadienol, 5,16-androstadien-3β-ol; DHA, dehydroepiandrosterone; androsterone, 3α-hydroxy-5α-androstan-17-one; aetiocholanolone, 3α-hydroxy-5β-androstan-17-one.

MATERIALS AND METHODS

The purification of solvents and preparation of alumina for chromatography have been described in a recent paper (Brooksbank & Gower, 1970). Thin-layer chromatography (TLC) was performed on glass plates coated with Kieselgel G (thickness 0·5 mm.). Purified methanol was allowed to run up the plates, which were dried before use. Gas–liquid chromatography (GLC) was performed using a Series 104, Model 24, dual flame ionization chromatograph (Pye–Unicam, Cambridge).

Source of blood and urine

After premedication with 750 mg. phencyclidine (Sernylan, Parke Davis & Co.) i.m., anaesthesia was induced in a sexually mature boar of proved fertility (age 3 yr.; approximate body weight 300 kg.) by open-mask administration of fluothane (Halothane, I.C.I. Ltd.) and oxygen which, after endotracheal intubation, was maintained with mixtures of fluothane and oxygen in a closed circuit rebreathing system. The right spermatic vein was exposed by mid-ventral laparotomy using sterile operative procedures, before an infusion of 20 μc [4-^{14}C]progesterone (58·5 mc/m-mole) into the substance of the right testicle. The results relating to this infusion will be published elsewhere. Venous blood and urine were obtained by catheterization of the right spermatic vein and bladder respectively. The animal was heparinized with 10,000 i.u. intravenously and blood was collected by continuous drainage into heparinized bottles, centrifuged at 3000 *g* for 15 min. and the plasma (143 ml.) removed. Both plasma and urine were stored at $-20°$.

Hydrolysis and extraction of urine and plasma

Plasma. To 143 ml. plasma were added 30 ml. of 2 N-NaOH and the mixture extracted with peroxide-free ether (3 × 50 ml.). The combined ether extracts were washed with water (50 ml.), then with 5 % acetic acid (50 ml.) and finally with water (2 × 50 ml.). After drying over anhydrous Na_2SO_4 the ether extract was filtered, the

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Na_2SO_4 washed with more ether and the extract evaporated to dryness under reduced pressure. This extract was designated the plasma F (free) fraction.

The aqueous portion which still contained steroid conjugates was adjusted to pH 4·5 with conc. HCl and 4 M-sodium acetate buffer (pH 4·5) was added. The mixture was incubated at 38° with 20 ml. Ketodase (William R. Warner & Co. Ltd., Eastleigh, Hants.) for 48 hr. Steroids released from glucuronides were then extracted with ether (3 × 50 ml.), the combined extracts washed with 10% $NaHCO_3$ solution (2 × 20 ml.) and then washed with water until neutral. After drying over Na_2SO_4 the ether was evaporated, as before, to give the plasma glucuronide (plasma G) fraction. Steroid sulphates remaining in the aqueous layer were hydrolysed at pH 0·8 and the liberated steroids continuously extracted with ether. The ether layer was separated, washed with $NaHCO_3$ solution and water as above. After drying and evaporating, a 'plasma sulphate' (plasma S) fraction was obtained.

Urine. Samples (200 ml.) were treated in the same way as plasma except that 2N-NaOH instead of 10% $NaHCO_3$ solution was used to clean the ether extracts obtained after hydrolysis of glucuronides and sulphates.

Column and thin-layer chromatography

Fractions F, G and S obtained from urine and plasma were chromatographed on alumina (5 g.), partially deactivated with water (4·5%, v/w, Gower & Ahmad, 1967). 16-Unsaturated steroids were eluted with 150 ml. of a mixture of benzene:light petroleum (80–100°) (1:1, v/v); the 17-oxosteroids, androsterone, aetiocholanolone and DHA were eluted in one fraction, after 16-unsaturated steroids, with 75 ml. of benzene containing ethanol (0·5%). This elution pattern had been checked in earlier experiments (Brooksbank & Gower, 1970).

In order to purify the plasma 16-unsaturated steroid fractions more thoroughly, they were submitted to thin-layer chromatography (TLC) and the plates developed three times with benzene:ether (9:1, v/v) (Gower, 1964). Three zones were removed from the plate as follows: Zone A, corresponding to the position of marker 5α-androstenone; Zone B, corresponding to the positions of androstadienone and 3α-androstenol; Zone C, corresponding to the positions of aetiocholenol, androstadienol and 3β-androstenol. Elutions of these and of a blank zone were performed as described earlier (Brooksbank & Gower, 1970).

Gas–liquid chromatography

Steroids were tentatively identified by GLC of free steroid and one or more derivatives on two or three columns, 5α-cholestane being used as internal standard. The columns used were: (i) cyclohexane dimethanol succinate (CHDMS, 0·6%) plus methyl polysiloxane gum (JXR, 0·75%) on 100–120 mesh silanized Chromosorb W; (ii) fluoralkyl silicone QF-1 (5%) on 100–120 mesh silanized Chromosorb W, and (iii) cyano alkyl silicone XE-60 (2·5%) on 80–100 mesh silanized Chromosorb G. Oven temperature was maintained at 196° and carrier gas flow rate at 50 ml./min.

Table 1. *Gas–liquid chromatography of C_{19}-16-unsaturated steroids from boar urine and spermatic vein plasma*

Source of compound		Column 1 (CHDMS/JXR)				Column 2 (XE-60)				Column 3 (QF-1)	
Extract	Authentic	Free steroid	Methyl oxime	CMDS ether	EMDS ether	Free steroid	Methyl oxime	CMDS ether	BMDS ether	Free steroid	CMDS ether
Plasma fraction F (TLC zone A) S	5α-Androstanone	0·254	0·250	—	—	0·772	0·344	—	—	—	—
		0·250	0·252	—	—	0·778	0·342	—	—	—	—
		—	—	—	—	0·72	—	—	—	—	—
	3α-Androstenol	0·228	—	0·402	0·532	0·343	—	0·610	0·760	0·325	0·610
	Androstadienone	0·357	—	—	—	1·182	—	—	—	1·24	—
Plasma fraction F (TLC zone B) S		0·230	—	0·396	—	0·360	—	—	—	—	—
		0·214	—	0·397	0·540	0·349	—	0·598	0·745	—	—
Urine fraction F		0·361	—	—	—	1·180	—	—	—	1·27	—
	Aetiocholenol	0·21	—	0·437	—	—	—	0·630	—	—	—
	Androstadienol	0·25	—	0·545	0·748	—	—	0·725	—	—	—
	3β-Androstenol	0·243	—	0·545	0·748	0·392	—	0·792	—	0·410	0·815
Plasma fraction F (TLC zone C) S		—	—	—	—	—	—	0·725	—	—	—
		—	—	0·543	0·750	—	—	0·795	—	—	—
Urine fraction G		0·247	—	0·548	—	0·393	—	0·796	—	0·403	0·800
Urine fraction S (unknown compound)		0·501	—	—	—	1·22	—	—	—	1·19	—

Boar urine and spermatic vein plasma were extracted with ether (see Methods) to give a free steroid (F) fraction. Conjugates remaining in the aqueous layer were hydrolysed separately and the steroids extracted with ether to give glucuronide (G) and sulphate (S) fractions. All fractions were chromatographed on alumina; plasma fractions were then separated by TLC and three zones (A, B and C) were eluted (see Methods). GLC was performed at 196° with a carrier gas flow rate of 50 ml./min. The values given are retention times relative to 5α-cholestane (= 1·00).

Cholestane times: Column 1, 21·5 min.; Column 2, 20·75 min.; Column 3, 18·3 min.

Boar urine and spermatic vein plasma were extracted with ether (see Methods) to give a free steroid (F) fraction. Conjugates remaining in the aqueous layer were hydrolysed separately and the steroids extracted with ether to give glucuronide (G) and sulphate (S) fractions. All fractions were chromatographed on alumina; plasma fractions were then separated by TLC and three zones (A, B and C) were eluted (see Methods). GLC was performed at 196° with a carrier gas flow rate of 50 ml./min. The values given are retention times relative to 5α-cholestane (= 1·00).
Cholestane times: Column 1, 21·5 min.; Column 2, 20·75 min.; Column 3, 18·3 min.

Preparation of derivatives

For 16-unsaturated ketones, such as 5α-androstenone, the *O*-methyl oxime was prepared by adding 50 μl. of a 2% (w/v) solution of methoxyamine hydrochloride in dry pyridine to the dried steroid and standing at room temperature overnight or at 37° for 2 hr. For 16-unsaturated alcohols such as androstenol, chloromethyldimethylsilyl (CMDS) ethers and bromomethyldimethyl silyl (BMDS) ethers were prepared by the method of Thomas & Walton (1968). Urinary and plasma 17-oxosteroids were analysed by GLC of their trimethylsilyl (TMS) ethers (Kirschner & Lipsett, 1963).

RESULTS

Spermatic vein plasma

The free steroid fraction, obtained from TLC zone A, contained a compound with a retention time relative to 5α-cholestane (RRT) identical with that of 5α-androstenone. It was possible to detect the penetrating odour of this compound on the

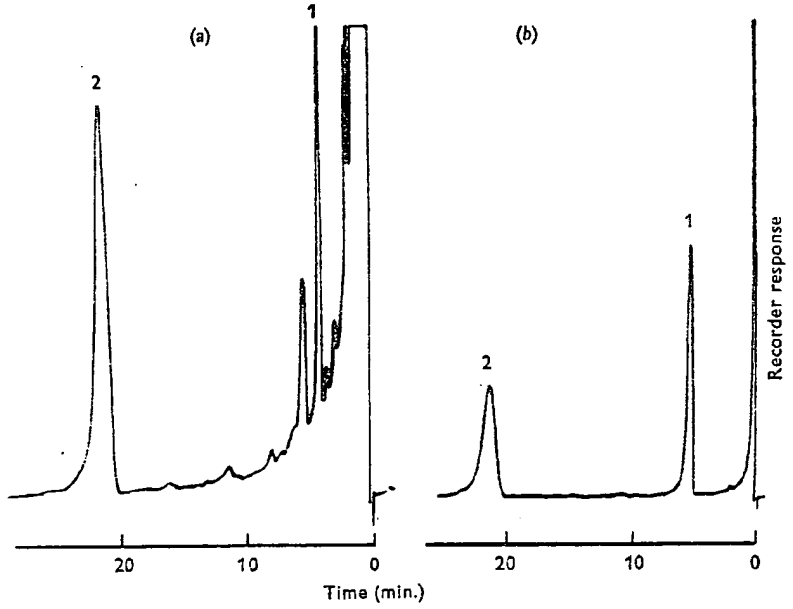

Fig. 1. (*a*) Gas–liquid chromatography (GLC) of thin-layer chromatography (zone A) extract converted to the *O*-methyl oxime (MO), obtained from the free steroid fraction of boar spermatic vein plasma (see text). (*b*) GLC of MO of 5α-androstenone (peak 1). 5α-Cholestane (peak 2) was added as internal standard. Column: CHDMS/JXR (0·60%/0·75%) at 196° with carrier gas flow 50 ml./min.

hot syringe needle after injection of an extract into the gas chromatograph. Definitive identification of this compound was obtained by combined gas chromatography–mass spectrometry (GC–MS) (see Appendix). GLC of the *O*-methyl oxime gave a peak with RRT identical with that of the authentic derivative (Table 1 and Fig. 1). No peak with the same retention time as that of the free ketone was detected when a

'blank' extract from TLC was chromatographed at the same sensitivity. GLC of TLC zone A extract, obtained from the plasma S fraction, showed no peaks which could be identified with 16-unsaturated steroids.

TLC zone B, obtained from the plasma F fraction, combined a trace of 3α-androstenol (Fig. 2*a*). In contrast, however, fraction S contained a relatively large amount

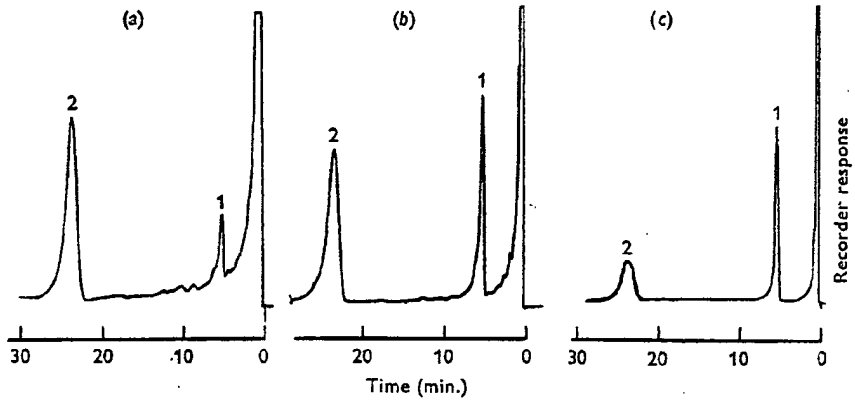

Fig. 2. Gas–liquid chromatography (GLC) of thin-layer chromatography (zone B) extracts obtained from (*a*) free steroid fraction and (*b*) sulphate fraction of boar spermatic vein plasma (see text). (*c*) GLC of authentic 3α-androstenol (peak 1). 5α-Cholestane (peak 2) was added as internal standard. Column conditions as in Fig. 1.

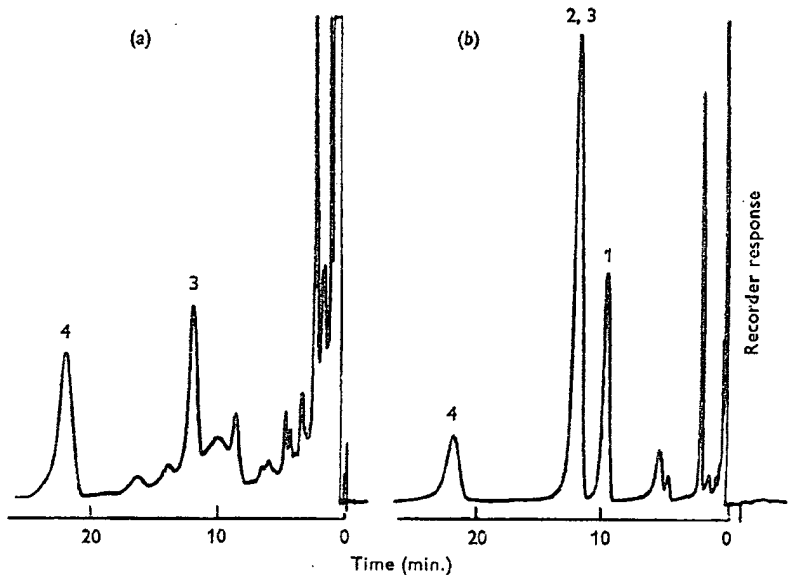

Fig. 3. (*a*) Gas–liquid chromatography (GLC) of thin-layer chromatography (zone C) extract, converted to the chloromethyldimethylsilyl (CMDS) ether, obtained from the sulphate fraction of boar spermatic vein plasma (see text). (*b*) GLC of a mixture of CMDS ethers of aetiocholenol (peak 1), androstadienol and 3β-androstenol (peaks 2, 3; subsequently resolved on XE-60, Table 1); 5α-cholestane (peak 4) was added as internal standard. Column conditions as in Fig. 1.

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of a compound, tentatively identified as 3α-androstenol, as free steroid and as CMDS and BMDS ethers (Fig. 2*b* and Table 1). Identification was again confirmed by GC–MS (see Appendix).

In a similar manner, TLC zone C, obtained from the plasma S fraction, was shown to contain a trace of androstadienol and a relatively large amount of 3β-androstenol (Fig. 3); identification of the latter was confirmed by GC–MS of the CMDS ether (see Appendix). GLC analysis of TLC zone C, obtained from the plasma F fraction, showed a small peak with RRT corresponding to that of 3β-androstenol and/or androstadienol. This, however, was not investigated further.

Preliminary GLC of the plasma glucuronide fraction did not show any peaks corresponding to 16-unsaturated steroids and this fraction was not therefore submitted to TLC.

Urine

GLC of the urinary '16-unsaturated steroid' fractions obtained from alumina showed a peak in the free steroid extract which had RRT identical with that of androstadienone on three columns (Table 1). However, the identification of this compound was not confirmed by GC–MS (see Appendix). No evidence was obtained for the presence of 5α-androstenone, which would be expected in the free steroid fraction, if present in urine.

Table 2. *C_{19}-16-unsaturated steroids identified in boar urine and spermatic vein plasma*

Fraction	Urine	Spermatic vein plasma
Free	None	5α-Androstenone; 3α-androstenol
Glucuronide	3β-Androstenol	None
Sulphate	None	3α-Androstenol; 3β-androstenol; androstadienol (trace)

Table 3. *17-Oxosteroids in boar urine and spermatic vein plasma*

	Urine (mg./l.)			Plasma (μg./100 ml.)		
Fraction	Androsterone	Aetio-cholanolone	DHA	Androsterone	Aetio-cholanolone	DHA
Free	Nil	Nil	0·06	Nil	—*	0·03
Glucuronide	1·75	2·76	6·57	Nil	Nil	0·2
Sulphate	Nil	Nil	4·18	—*	—*	12·0

* Not determined.

In the glucuronide fraction there was a compound which, as CMDS ether, had the same RRT on three columns as authentic 3β-androstenol (Table 1). Assuming 85–90% recovery of 16-unsaturated steroids, as obtained from human urine (Brooksbank & Gower, 1970), the concentration of this compound was approx. 250 μg./l. Confirmatory identification of this compound was obtained by GC–MS (see Appendix). No evidence was obtained for the presence of other 16-unsaturated alcohols, such as 3α-androstenol, which by comparison with human urine (Brooksbank & Gower, 1970) might be expected to occur in the glucuronide fraction.

The sulphate fraction contained a compound with RRT similar to that of androstadienone on two of the columns used (Table 1). GC–MS results for this compound have not conclusively established its identity (see Appendix).

A summary of the 16-unsaturated steroids found in the spermatic vein plasma and urine of the boar is given in Table 2.

Urine and plasma 17-oxosteroids

The results of these analyses are given in Table 3.

DISCUSSION

C_{19}-16-unsaturated steroids in boar spermatic vein plasma and in urine

The results reported here demonstrate the presence of 5α-androstenone, 3α-androstenol and 3β-androstenol in boar spermatic vein plasma. Further experiments are in progress to estimate the testicular production rate of these compounds from measurements of their arterio-venous differences and of testicular blood flow. There is already some evidence to suggest that these compounds have a testicular origin. Both 3α- and 3β-androstenols have been isolated from pig testes (Prelog & Ruzicka, 1944) and, together with 5α-androstenone, they are known to be synthesized by the same tissue *in vitro* from C_{21}-precursors, pregnenolone and progesterone (Gower & Ahmad, 1967; Ahmad & Gower, 1968; Katkov & Gower, 1968). In the present experiment 3α- and 3β-androstenols were found to occur in plasma predominantly as sulphates with only traces as free steroids. This is in keeping with what is known of alcohols such as DHA or androsterone which occur predominantly as sulphates in human plasma and, according to the present results (Table 3), also in boar spermatic venous plasma.

Only two reports have appeared concerning the presence of 16-unsaturated steroids in human blood. In human male peripheral plasma androstadienone occurs mostly in the 'sulphate conjugate' fraction and to a lesser extent as free steroid (Brooksbank *et al.* 1969) and 3α-androstenol has been tentatively identified in the peripheral plasma of a woman with a virilizing adrenal carcinoma. After removal of the tumour, this compound was undetectable (Gower & Stern, 1969).

The finding of 5α-androstenone in spermatic venous plasma may be related to its presence in boar fat (Patterson, 1968*a*). This compound is believed to be responsible for the strong taint of boar meat. Furthermore, it seems likely that the ketone is present in peripheral plasma and diffuses into fat because of its high lipid solubility. Although the presence of the same ketone in boar saliva has been demonstrated already (Patterson, 1968*b*), no data are yet available as to the relative concentrations in saliva and peripheral plasma.

The present experiments show that the boar excretes 3β-androstenol conjugated with glucuronic acid whereas in man 3α-androstenol is excreted in the glucuronide fraction (Brooksbank & Haslewood, 1961; Cleveland & Savard, 1964; Brooksbank & Gower, 1970). No urinary 16-unsaturated steroid sulphates have been found in either species (Brooksbank & Gower, 1964) except in the case (mentioned above) of a woman with a virilizing adrenal carcinoma where the urinary 3α-androstenol excretion rate was higher than normal (Gower & Stern, 1969).

The physiological role of 5α-androstenone and other 16-unsaturated steroids is not completely clear, but there is evidence to show that both the ketone and 3α-androstenol can act as pheromones. Thus Patterson (1968*b*) suggested that when the boar becomes sexually excited and salivates profusely, the pungent smell of 5α-androstenone on the animal's breath may be responsible for inducing a sow *in oestrus* to adopt the mating stance. Recent work (Melrose *et al.* 1968) has shown that over 50% of maiden gilts, which did not respond to the conventional back-pressure test for the detection of oestrus *in the absence of a boar*, responded immediately when 5α-androstenone was sprayed into the atmosphere from an acrosol.

Urinary and spermatic vein plasma 17-oxosteroids

The results obtained in this paper by column, thin-layer and gas–liquid chromatography are essentially in agreement with those obtained by earlier workers who used column and paper chromatographic methods for separation and the Zimmermann method for analysis. DHA appears to be the most important urinary neutral 17-oxosteroid (Huis in't Veld, Louwerens & Reilingh, 1964; Clark, Raeside & Solomon, 1965) and is now known to be of testicular, rather than of adrenal, origin in the boar. Moreover, DHA is the precursor of urinary 17-oxosteroids rather than testosterone (Huis in't Veld *et al.* 1964; Clark *et al.* 1965; Huis in't Veld, 1968). In contrast to DHA, the urinary excretion rates of androsterone and aetiocholanolone in the boar are relatively small (Table 3).

The amounts of DHA both as sulphate and as free steroid in spermatic venous blood (Table 3) are in accord with the results of Baulieu, Fabre-Jung & Huis in't Veld (1967). These workers also showed that the concentration of DHA in spermatic venous blood was greater than that in the spermatic arterial blood or in the peripheral blood—a finding consistent with the testicular origin of DHA in the boar.

D. B. G. thanks Mr D. C. Bicknell for excellent technical assistance, Mr L. Armitage for photographing the GLC tracings and Guy's Hospital Endowments Fund for financial support.

APPENDIX

IDENTIFICATION OF C_{19}-16-UNSATURATED STEROIDS IN BOAR URINE AND SPERMATIC VEIN PLASMA BY COMBINED GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

D. B. Gower* and R. L. S. Patterson†

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The free steroids and CMDS ethers of the urine F, G, and S and plasma F and S fractions, isolated and prepared as described in the main text, were examined by gas chromatography–mass spectrometry (GC–MS).

The analyses were carried out in an L.K.B. 9000 GC–MS equipped with a 1·5 m. × 3 mm. i.d. glass chromatography column packed with 3% XE-60 on Chromosorb W (80–100 mesh). The column was maintained at 200°, the injection block at 190° and the molecular separator at 200°. The flow of carrier gas (helium) was 32 ml./min.

Mass spectra were recorded at 70 ev and the range m/e 0–400 was scanned in 8 sec. Background spectra were recorded immediately before or after the spectrum of each compound to allow peaks arising from the stationary phase bleed or from preceding compounds to be identified and subtracted from the compound spectrum. Line diagrams, normalized to the base peak, were prepared from the corrected spectra of the reference and experimental compounds before comparison. Reference spectra of 5α-androstenone, 3α- and 3β-androstenols have already been published (Patterson, 1968*a*, *b*).

Spermatic vein plasma. The spectrum obtained for the major compound in the plasma F fraction, TLC zone A, was identical with the spectrum of authentic 5α-androstenone.

3α-Androstenol was confirmed as a minor component of the plasma F fraction and as a major component of the plasma S fraction, TLC zone B. The spectrum of the free steroid, obtained from both samples, agreed well with that of the authentic compound.

The identity of 3β-androstenol in the plasma S fraction, TLC zone C, was confirmed by comparison of the spectrum of its CMDS ether with that of an authentic sample (Fig. 4). The lack of minor peaks between m/e 107 and 173 may be explained on the basis of over-correction of the sample spectrum for background from stationary phase bleed and/or from preceding compounds. This is borne out by the fact that the relative abundance of many peaks in the spectrum of the unknown is lower than that for the corresponding peaks obtained from the authentic compound.

Urine. The compound in the urine F fraction which had the same relative retention to cholestane as androstadienone on the three GLC columns used, gave a mass spectrum completely different from that of authentic androstadienone. Although the sample spectrum was weak, none of the predominant ions (270 (M^+), 255, 228, 146, 105, 91, 79, 41) of the androstadienone spectrum was detected. The isolated compound therefore was not androstadienone, despite the very good agreement of the GLC retention results (Table 1). This conclusion was confirmed by re-examination of the urine F fraction by TLC using benzene:ether (9:1, v/v) as solvent. The eluate of the zone corresponding to the position of androstadienone showed on GLC no peak at the correct retention time for the compound.

A strong spectrum was obtained for 3β-androstenol in the hydrolysed urine glucuronide fraction which confirmed the tentative identification by GLC of both the free alcohol and its CMDS ether (Table 1).

The shape of the GLC peak for the component in the urine S fraction suggested that more than one compound might be present, but four scans taken during elution of the peak showed that it was either homogeneous or composed of isomers. No significant differences were found in the spectra, and m/e 279 was the ion of greatest mass recorded in each spectrum, except for the small peak at m/e 280, which was most probably the ^{13}C isotope contribution. These spectra were very similar to the spectrum obtained for the unidentified compound originally thought to be androstadienone in the urine F fraction. The same major ions were present in both spectra

although differences occurred in the minor peaks. The compounds have not been identified although it is suggested that they may not be steroids and are esters of phthalic acid. Phthalic acid esters are known to occur as impurities in solvents and some of the major spectral peaks can be accounted for in this way. Several of the

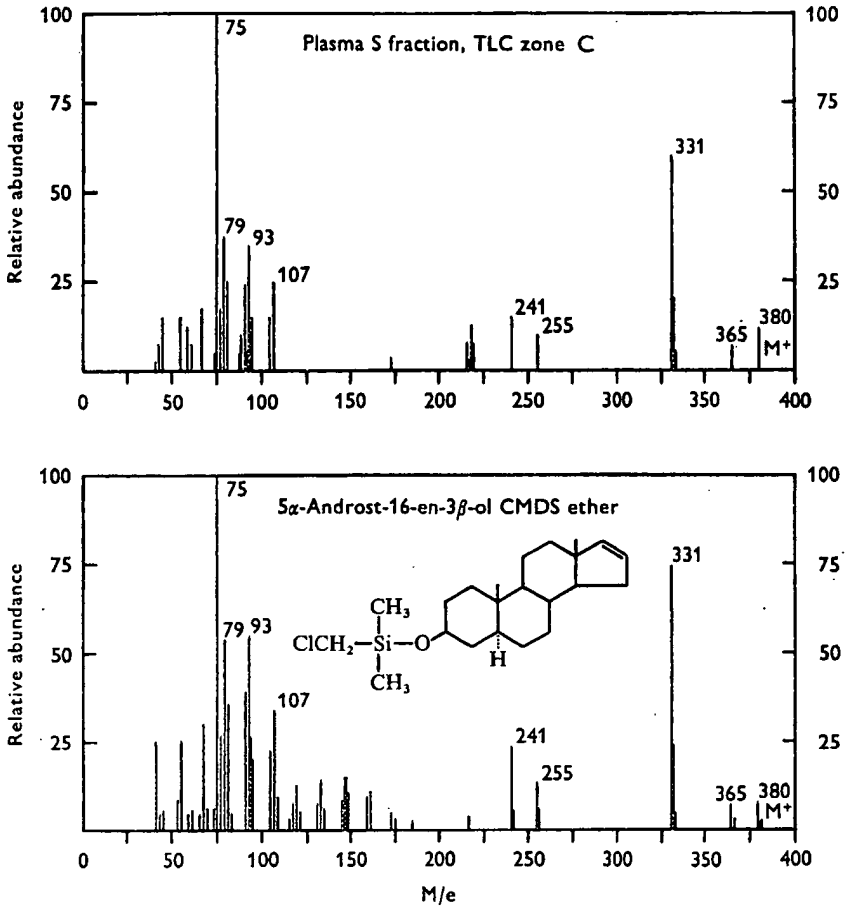

Fig. 4. Top: mass spectrum of thin-layer chromatography (TLC) (zone C) extract, as chloromethyldimethylsilyl (CMDS) ether, obtained from the sulphate fraction of boar spermatic vein plasma (see text). Bottom: mass spectrum of the CMDS ether of authentic 3β-androstenol.

fragment ions recorded for the unknown agree with those reported (Cornu & Massot, 1966) for di-*n*-octylphthalate, although they differ considerably in relative abundance. However, the major peaks m/e 279, 113, 104 and 83 are not accounted for, nor is the apparent absence of m/e 57 and 108, reported as present in the mass spectrum of di-*n*-octyl-phthalate.

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STUDIES IN STEROID METABOLISM

XXV. ISOLATION AND CHARACTERIZATION OF NEW URINARY STEROIDS*

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Two steroids hitherto undescribed, 3α,17α-dihydroxyallopregnane-20-one (I) and pregnane-3α,11β,20α-triol (II), have been isolated from urine, characterized, and identified by partial synthesis. In addition to these, the known compounds, 3α,11β-dihydroxypregnane-20-one (III) (1), 3α-hydroxy-Δ^{16} pregnene-20-one (IV) (2), and 3β-hydroxy-Δ^5-androstene-7,17-dione (V) (3), have been identified for the first time as constituents of the ketosteroid fraction of urine. I was identified in the "α-ketosteroid" fraction from the urine of patients with adrenal hyperplasia. The amount present was usually smaller than the epimeric steroid, 3α,17α-dihydroxypregnane-20-one, although both metabolites may be excreted in relatively large amounts by patients with this adrenal disorder. The triol II and the closely related ketone, 3α,11β-dihydroxypregnane-20-one (III), were obtained from the urine of patients treated with large amounts of corticosterone as a therapeutic measure. The fourth urinary steroid, 3α-hydroxy-Δ^{16}-pregnene-20-one (IV), was detected in the urine of a patient with the classic symptoms of Cushing's syndrome. The compound was unquestionably identified from its infra-red spectrum; the amount present was so small that isolation in crystalline form and identification by mixture melting point and comparison of physical constants were precluded. 3β-Hydroxy-Δ^5-androstene-7,17-dione (V) has been found in many urines from both normal and abnormal subjects; it is excreted in larger amount in adrenal abnormalities, especially those characterized by increased excretion of dehydroisoandrosterone. This compound was previously reported as an unknown β-ketosteroid, Compound B6, by Lieberman, Dobriner, Hill, Fieser, and Rhoads (4).

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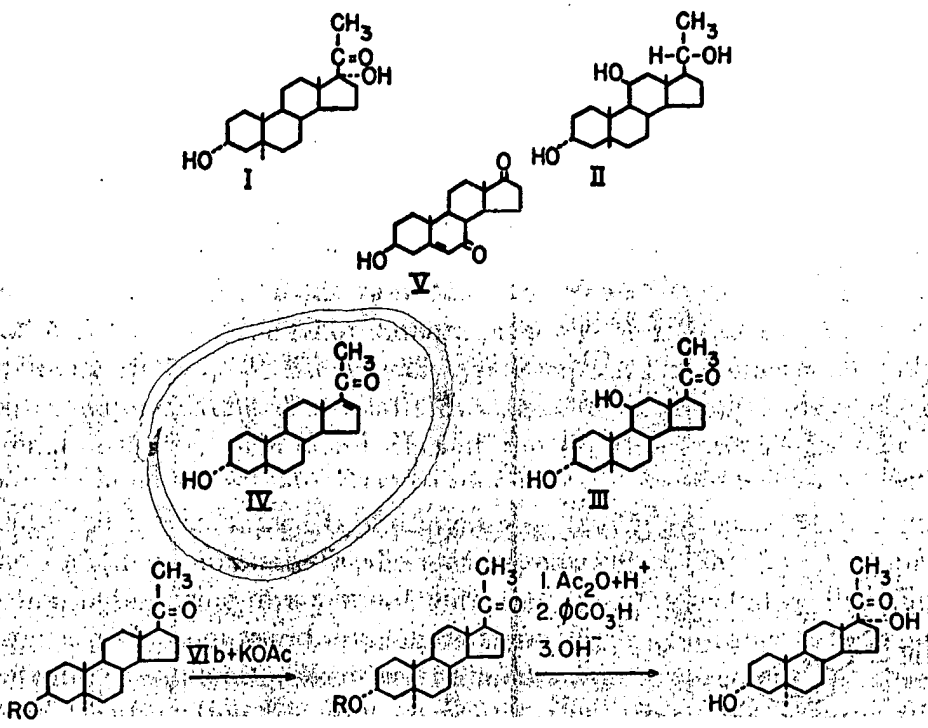
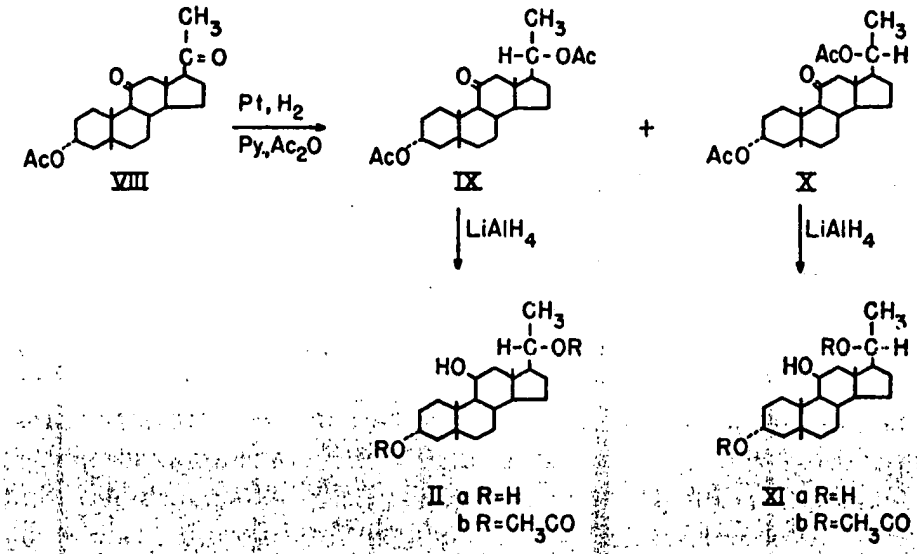
The partial synthesis of 3α,17α-dihydroxyallopregnane-20-one (I) was achieved by the application of the method of Kritchevsky and Gallagher (5) to 3α-hydroxyallopregnane-20-one (VIIa). VIIb was prepared from 3β-hydroxyallopregnane-20-one (VIa) by formation of the 3-tosylate (VIb), followed by replacement of the tosyloxy group with acetate by heating with potassium acetate in acetic acid. The resultant 3α-acetoxyallopregnane-20-one (VIIb) was isolated as the free hydroxy ketone (VIIa) and converted to the 17,20-enoldiacetate upon treatment with acetic anhydride



in the presence of a catalytic amount of *p*-toluenesulfonic acid. Oxidation of the enolacetate with perbenzoic acid followed by alkaline hydrolysis yielded 3α,17α-dihydroxyallopregnane-20-one (I).

Pregnane-3α,11β,20α-triol (II) was prepared from 3α-acetoxypregnane-11,20-dione (VIII). Catalytic reduction of VIII yielded a mixture of the 20α (IX) and 20β (X) epimers of 3α-acetoxy-20-hydroxypregnane-11-one. The mixture was acetylated and the epimers were separated (6). 3α,20α-Diacetoxypregnane-11-one (IX) was readily converted to pregnane-3α,-11β,20α-triol (IIa) by reduction with lithium aluminum hydride. The free triol was converted to the 3,20-diacetate IIb, which was easily purified and exhibited excellent physical constants. Lithium aluminum hydride

reduction of pregnane-3α,20β-diacetoxypregnane-11-one (X) yielded pregnane-3α,11β,20β-triol (XIa), characterized as both the free compound and the 3,20-diacetate XIb. This substance has not as yet been encountered in extracts of urine, but its presence may possibly be disclosed by further investigation.



EXPERIMENTAL

All melting points are corrected. The optical rotations were taken in chloroform unless otherwise specified.

3α,17α-Dihydroxyallopregnane-20-one (I); Isolation—This compound was isolated from the urine of two patients with adrenal hyperplasia. Partition chromatography of the "α-ketonic" fraction on silica gel with ethanol-methylene chloride (7) gave 8.0 mg. of crystalline material. The infra-red spectrum was almost identical with that of an authentic sample of 3α,17α-dihydroxyallopregnane-20-one. Paper partition chromatography for 24 hours of a small portion with the system 1:1 propylene glycol-methanol and 1:1 cyclohexane-toluene showed that the above 3α,17α-dihydroxyallopregnane-20-one contained a faint trace (less than 1 per cent) of 3α,17α-dihydroxypregnane-20-one. The product was digested with cyclohexane and twice recrystallized from benzene to yield 2 mg. of 3α,17α-dihydroxyallopregnane-20-one (I), m.p. 215–216.5°; there was no depression of the melting point on admixture with tl. synthetic sample, and there was a depression on admixture with 3α,17α-dihydroxypregnane-20-one. The infra-red spectrum was identical with that of the synthetic product.

Synthesis of 3α-Hydroxyallopregnane-20-one (VIIa)—A cold solution of 10 gm. of *p*-toluenesulfonyl chloride in 15 ml. of pyridine was added to a cold solution of 3.0 gm. of 3β-hydroxyallopregnane-20-one (VIa) in 30 ml.

of pyridine, and the mixture was allowed to stand for 3 days at room temperature. The solution was poured into ice and water and the resulting precipitate collected by filtration. The semicrystalline material was dissolved in ethyl acetate and washed with dilute acid, dilute base, and water. After drying the organic solution, the solvent was evaporated to give 3.85 gm. of yellowish oil. Recrystallization from ether gave 3.10 gm. of white crystalline product. Further recrystallization from acetone-petroleum ether afforded 2.26 gm. of 3β-*p*-toluenesulfonoxyallopregnane-20-one (VIb), m.p. 133–135°. The analytical sample after recrystallization from cyclohexane melted at 133.5–135°, $[\alpha]_D^{25}$ +58.3°.

$C_{28}H_{40}O_4S$. Calculated, C 71.18, H 8.47; found, C 71.50, H 8.25

A solution of 2.1 gm. of 3β-*p*-toluenesulfonoxyallopregnane-20-one and 2 gm. of freshly fused potassium acetate in 50 ml. of glacial acetic acid was refluxed for 3 hours. The reaction mixture was diluted with 10 per cent sodium chloride solution and extracted with ether. The ether solution was washed repeatedly with dilute sodium hydroxide solution and water and dried over sodium sulfate. Evaporation of the solvent gave 1.46 gm. of oil that crystallized on standing. The acetolysis product was hydrolyzed at room temperature for 30 minutes with 32 ml. of ethanol and 3 ml. of 20 per cent sodium hydroxide solution. The product (1.37 gm.) was chromatographed on silica gel to yield 525 mg. of Δ^2-allopregnene-20-one, which on recrystallization from methanol melted at 126–129°; 330 mg. of a mixture of Δ^2-allopregnene-20-one and 3α-acetoxyallopregnane-20-one; 505 mg. of 3α-hydroxyallopregnane-20-one (VIIa), which on recrystallization from acetone-petroleum ether melted at 174–175°; reported, 176° (8) and 176–178° (9). Δ^2-Allopregnene-20-one has been previously isolated from urine extracts by Lieberman *et al.* (4).

3α,17α-Dihydroxyallopregnane-20-one (I) and 3-Monoacetate—Δ^{17}-Allopregnene-3α,20-diol diacetate was prepared by slow distillation of a solution of 200 mg. of 3α-hydroxyallopregnane-20-one (VIIa) and 100 mg. of *p*-toluenesulfonic acid in 30 ml. of acetic anhydride (5). The reaction mixture was taken up in ether and washed with cold sodium carbonate solution and water. The ether solution was dried and the solvent evaporated. The residue was dissolved in a liter of petroleum ether and poured through a short alumina column. Upon removal of the solvent under diminished pressure, 224 mg. of crystalline Δ^{17}-allopregnene-3α,20-diol diacetate were obtained. Without further purification, the enol acetate was treated overnight at room temperature with 5 ml. of 0.49 M perbenzoic acid in benzene. Ethyl acetate was added to the reaction mixture, and the solution was washed with sodium carbonate solution and with water. After removal of the solvent, the crude 17α,20-epoxyallopregnane-3α,20-diol diacetate was

saponified with 1 N aqueous ethanolic sodium hydroxide solution at room temperature for 3.5 hours. Purification by chromatography on silica gel and recrystallization from acetone and benzene afforded 3α,17α-dihydroxyallopregnane-20-one (I), m.p. 214–216.5°, $[\alpha]_D^{27}$ +45.2° (ethanol).

$C_{21}H_{34}O_3$. Calculated, C 75.40, H 10.24; found, C 75.33, H 10.46

Acetylation with acetic anhydride and pyridine at room temperature for 4.5 hours yielded 3α-acetoxy-17α-hydroxyallopregnane-20-one, m.p. 216.5–220°, $[\alpha]_D^{27}$ +1.7°.

$C_{23}H_{36}O_4$. Calculated, C 73.40, H 9.57; found, C 73.25, H 9.52

Pregnane-3α,11β,20α-triol (IIa) and 3,20-Diacetate (IIb); Isolation—The compound (IIa) was isolated from the urine of a patient with lymphatic leucemia who had received 800 mg. of corticosterone orally each day. Partition chromatography of the non-ketonic fraction on silica gel with ethanol-methylene chloride (7) yielded crystalline fractions whose infra-red spectrum was that of pregnane-3α,11β,20α-triol. Acetylation of the products yielded 140 mg. which was recrystallized from petroleum ether, from benzene, and from acetone-petroleum ether to give 23 mg. of pregnane-3α,11β,20α-triol 3,20-diacetate (IIb), m.p. 191–192°; there was no depression of the melting point on admixture with the synthetic sample; the infra-red spectrum was identical with that of the synthetic sample.

Synthesis—A solution of 237 mg. of 3α,20α-diacetoxypregnane-11-one (IX) (6) in 20 ml. of anhydrous benzene and 30 ml. of anhydrous ether was added dropwise with stirring to a solution of 300 mg. of lithium aluminum hydride in 75 ml. of anhydrous ether. After the reaction was complete, the mixture was heated under reflux for 1.5 hours. The excess lithium aluminum hydride was destroyed with ethyl acetate and dilute acid. The organic layer was separated and washed with sodium carbonate and water. The solvent was removed and 198 mg. of product were obtained. Recrystallization from ethyl acetate and from acetone gave a triol (IIa), m.p. 204–205°, $[\alpha]_D^{24}$ +40.6°. Acetylation with acetic anhydride and pyridine at room temperature resulted in 132 mg. of product. Recrystallization from petroleum-ether gave pregnane-3α,11β,20α-triol 3,20-diacetate (IIb), m.p. 192–192.5°, $[\alpha]_D^{29}$ +56.3°.

$C_{25}H_{40}O_6$. Calculated, C 71.39, H 9.59; found, C 71.20, H 9.62

Pregnane-3α,11β,20β-triol (XIa) and 3,20-Diacetate (XIb)—A solution of 3.7 gm. of 3α,20β-diacetoxypregnane-11-one (X) (6) in 175 ml. of a benzene-ether mixture was added dropwise with stirring to a suspension of 3 gm. of lithium aluminum hydride in 25 ml. of anhydrous ether. The mix-

ture was heated under reflux for 2 hours, and the excess reagent was destroyed with ethyl acetate and dilute acid. The organic layer was washed with base and water. The solvent was removed and 3.07 gm. of triol were obtained. Repeated digestion with acetone gave 2.69 gm. of pregnane-3α,11β,20β-triol (XIa); m.p. 232–234°. The analytical sample melted at 233.5–234°.

$C_{21}H_{36}O_3$. Calculated, C 74.95, H 10.78; found, C 75.07, H 10.73

Acetylation of the triol with pyridine and acetic anhydride at room temperature gave pregnane-3α,11β,20β-triol 3,20-diacetate (XIb); m.p. 121.5–123.5°, $[\alpha]_D^{20}$ +70.7°.

$C_{25}H_{40}O_5$. Calculated, C 71.39, H 9.59; found, C 71.30, H 9.49

3α,11β-Dihydroxypregnane-20-one (III); Isolation—This compound was isolated from the urine of two patients with lymphatic leucemia who received 600 to 800 mg. of corticosterone orally each day. Chromatography of the "α-ketonic" fraction on silica gel with ethyl acetate-petroleum ether as eluent gave 44 mg. of oily material, the infra-red spectrum of which indicated the presence of 3α,11β-dihydroxypregnane-20-one. Further purification of this material by partition chromatography on silica gel with ethanol and methylene chloride gave 8 mg. of a colorless oil. After three crystallizations from ethyl acetate 2 mg. of 3α,11β-dihydroxypregnane-20-one as prisms, m.p. 212–217°, were obtained; the admixture with authentic material (10), m.p. 220–222°, melted at 216–220°; the infra-red spectrum was indistinguishable from that of an authentic sample.

3α-Hydroxy-Δ^{16}-pregnene-20-one (IV)—The compound was found in the urine of a patient with Cushing's syndrome. In the partition system with ethanol on silica gel, and methylene chloride-2 per cent. ethanol as the eluent, the compound was detected immediately before 11β-hydroxy-Δ^4-androstene-3,17-dione (11). Infra-red spectrometry of the free steroid revealed the presence of a hydroxyl group and a double bond and a band at 1670 cm.$^{-1}$ characteristic of the Δ^{16}-20-ketone system; the acetylated product exhibited acetate absorption at 1739 and 1236 cm.$^{-1}$, the characteristic band at 1670 cm.$^{-1}$, and no evidence of a free hydroxyl function. The spectra of the free steroid and the acetate were in all essential details identical with the authentic samples obtained from Dr. Marker (2).

3β-Hydroxy-Δ^5-androstene-7,17-dione (V); Isolation—This compound was isolated from the urine of several patients with adrenocortical disorders, including both tumors and hyperplasia. Chromatography of the "β-ketonic" fraction on magnesium silicate-Celite gave eluates whose infra-red spectra indicated the presence of 3β-hydroxy-Δ^5-androstene-7,17-dione. Recrystallization from benzene-petroleum ether and from ethyl acetate yielded

needles, m.p. 245–248°, $[\alpha]_D^{23}$ −66.4° (methanol). The mother liquors were combined and rechromatographed on silica gel. The fractions containing V, as indicated by infra-red spectrum, were combined and acetylated with acetic anhydride and pyridine at room temperature. Recrystallization from methanol gave needles of 3β-acetoxy-Δ^5-androstene-7,17-dione, m.p. 184.5–187.5°, $[\alpha]_D^{25}$ −72.0° (ethanol), ϵ_{2350} = 13,800 (ethanol); reported, m.p. 184°, $[\alpha]_D^{24}$ −74° (alcohol) (3); log ϵ_{2350} = 4.09 (12); the infra-red spectrum was identical with that of an authentic sample.

DISCUSSION

Two of the metabolites of corticosterone reported in this study have undergone further reduction in the side chain, with the loss of the characteristic ketol in addition to the usual saturation of the α,β-unsaturated ketone system in ring A. The reductive removal of the C-21 hydroxyl group from steroids containing the dihydroxyacetone side chain has been reported earlier from these laboratories (13). The total process is analogous to the previously observed conversion of desoxycorticosterone to pregnane-3α,20α-diol (14) and of dehydrocorticosterone to 11-ketopregnane-3α,20α-diol (15). However, it is important to note that, in the transformation of corticosterone to the triol II and to the dihydroxy ketone III, the hydroxyl function at C-11 has been unaltered in the process. The retention of the 11-oxygen function, despite extensive transformation elsewhere in the molecule, has been frequently noted, and the results of this study confirm the generalization of this reaction.

With the synthesis of 3α,17α-dihydroxyallopregnane-20-one, all four isomers of this compound, epimeric at C-3 and C-5, have now been described. Only two of these have been discovered in human urine, and both were found in the relatively well explored α-ketosteroid fraction. The remaining two isomers, 3β,17α-dihydroxyallopregnane-20-one (Reichstein's Substance L) and 3β,17α-dihydroxypregnane-20-one, should on the basis of biochemical analogy be expected in the β-ketosteroid fraction. Efforts toward the isolation and identification of these compounds have been initiated.

The identification of 3α-hydroxy-Δ^{16}-pregnene-20-one in human urine presents a new problem as to whether the substance was excreted as such or was derived, as an artifact of hydrolysis or separation, from a precursor such as 3α,16?-dihydroxypregnane-20-one. For the time being this must remain an open question, since the ready interconvertibility of these compounds has been well established (16). It is noteworthy that the epimeric substance 3β-hydroxy-Δ^{16}-allopregnene-20-one has been isolated from the urine of pregnant mares by Klyne, Schachter, and Marrian (17) by procedures which would not preclude the initial presence of a 16-hydroxyl group.

A similar though less strong reservation must be made about the significance of the fifth compound 3β-hydroxy-Δ⁵-androstene-7,17-dione. From the work of Bergström and Wintersteiner (18), it has been well established that Δ⁵-steroids are capable of facile autoxidation at C-7, with the formation of α,β-unsaturated ketones of the type present in this metabolite. It is therefore possible that this substance may have arisen from the oxidation of the commonly present dehydroisoandrosterone, but, in the absence of proof, it seems more reasonable to accept the positive attitude that the compound is in fact a metabolite. It is felt that the increased amount of this substance in adrenal disorders is suggestive evidence for this interpretation.

SUMMARY

Five new steroid hormone metabolites have been isolated from urine. Two of these were the hitherto undescribed 3α,17α-dihydroxyallopregnane-20-one and pregnane-3α,11β,20α-triol. The structures were established by partial synthesis of the compounds. The other three metabolites were 3α,11β-dihydroxypregnane-20-one, 3α-hydroxy-Δ¹⁶-pregnene-20-one, and 3β-hydroxy-Δ⁵-androstene-7,17-dione. The preparation of Δ²-allopregnene-20-one and pregnane-3α,11β,20β-triol has been described.

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Thank you

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Hydrolysis of Ketosteroid Hydrogen Sulfates by Solvolysis Procedures*

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Because of the sensitivity of some steroid sulfates to acid and to heat, mild methods of hydrolysis for these conjugates have been continuously sought. Alone among those chemical methods which have been suggested (1), the procedure consisting f the exhaustive continuous extraction of appropriately acidified aqueous solutions of these conjugates with ether (2, 3) has found wide use since it is practical and does not result in artifact f rmation. Although this method has been successfully applied to urines and to blood (4), the mechanism of this hydrolysis has not been well understood. That this type of hydrolysis does not occur as a result of simple displacement of the equilibrium, $ROSO_3H + H_2O = ROH + H_2SO_4$ by the removal of the steroid alcohol from the aqueous phase has already been suggested (1). When an aqueous solution of PDS,[1] pH 1 with H_2SO_4, was allowed to remain at room temperature overnight, no hydrolysis occurred despite the enormous excess of water and the insolubility of the product. Subsequent experience (see below) revealed that an acidified solution of PDS remained unchanged on standing for 9 days at room temperature. The observation that substitution of benzene for ether in the continu us extraction of the acidified solution of PDS did not result in hydrolysis confirmed that equilibrium factors were not of principal concern.

It is the purpose of this paper to report our efforts to elucidate the mechanism of the two-phase hydrolytic procedure of ketosteroid sulfates and to describe mild hydrolytic procedures for these urinary conjugates which do not require continuous extraction apparatus. The application of these procedures to human urine is also illustrated.

EXPERIMENTAL

All solvents used were Mallinckrodt, analytical grade, and were redistilled without further purification with the exception of the ether which was redistilled over KOH before use. The latter procedure gives peroxide-free ether, as determined with acidified KI or titanium sulfate solution. PDS was prepared by treating dehydroisoandrosterone in pyridine with a suspension of pyridine sulfurtrioxide according to the method of Fieser (5). It melted at 219° to 223° (corrected) after recrystallization from methanol (6). It was analyzed for K, as follows:

$C_{19}H_{27}O_5SK$

Calculated: K, 9.55
Found: K, 9.02[2]

The rate of hydrolysis of DHS was followed by determining the concentration of the liberated steroid alcohol. The free steroid was separated from the unchanged sulfate by distributing the contents of the reaction mixtures under study between one of the following two-phase systems: benzene:H_2O, chloroform:H_2O, methylene chloride:H_2O, or ethyl acetate:H_2O. The sulfate ester remains in the aqueous phase while the free steroid is quantitatively extracted by the organic phase. A quantitative separation of DHS from dehydroisoandrosterone was accomplished by plunging aliquots of the reaction mixture, at appropriate time intervals, into one of the above mentioned organic solvents in glass-stoppered tubes and shaking with an equal volume of H_2O or 1 per cent $NaHCO_3$ solution. Aliquots of the organic phase were then filtered through filter paper and evaporated to dryness under a stream of nitrogen. The dehydroisoandrosterone content of the residues was determined by the Holtorff-Koch modification of the Zimmermann reaction (7).

The partition of PDS between the two-phase systems listed in Table 1 was determined by a technique previously described (8). Aqueous solutions of PDS were shaken in glass-stoppered tubes with organic solvents. Appropriate aliquots of the organic phase were withdrawn and were evaporated to dryness following the addition of a few drops of pyridine. The addition of pyridine was necessary since evaporation of a solution of the free $ROSO_3H$ to dryness results in decomposition with a consequent lowering of the Zimmermann titer. Solutions of the pyridinium salts are stable and may be evaporated without difficulty.

Urinary extracts were chromatographed by the gradient elution technique on alumina (9). Carbon disulfide soluti ns of the contents of the eluates comprising a chromatographic peak were identified by their infrared spectra using a model 12 Perkin-Elmer infrared spectrometer.

RESULTS

I. Model Experiments on the Hydrolysis of DHS

The following experiments were designed to demonstrate that the two-phase ether hydrolysis occurs in the ether phase and does not result from a displacement f equilibrium.

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[1] The abbreviations used are: PDS, potassium dehydroisoandrosterone sulfate; DHS, dehydroisoandrosterone hydrogen sulfate.

[2] This analysis was carried out by Mr. J. Alicin, Metuchen, New Jersey.

TABLE I

The partition of potassium dehydroisoandrosterone sulfate between various two-phase systems

No.	Solvent system	Vol. of each phase	C_{org}* in μg./ml.
1	0.1 N H_2SO_4:EtOAc	4	68† (K = 0.4)‡
2	1.0 N H_2SO_4:EtOAc	4	190† (K = 3.4)
3	2.0 N H_2SO_4:EtOAc	4	232†
4	0.15 M NaCl:EtOAc	4	16†
5	1.5 M NaCl:EtOAc	4	112† (K = 0.9)
6	3.0 M NaCl:EtOAc	4	240†
7	3.0 M NaCl:EtOAc 0.1N H_2SO_4	4	244†
8	2.0 N H_2SO_4:CH_2Cl_2	4	1.6§
9	2.0 N H_2SO_4:$CHCl_3$	50	2.0§
10	2.0 N H_2SO_4:Benzene	‖	0.3§
11	0.1 N H_2SO_4:Ether	50	4.7§
12	2.0 N H_2SO_4:Ether	50	31.0§ (K = 0.3)

* C_{org}, concentration in organic phase; C_{aq} concentration in aqueous phase.

† C_{aq} before distribution = 245 μg./ml.

‡ K, partition coefficient = C_{org}/C_{aq}. C_{aq} is calculated by difference. In those instances where C_{org} was very large or very small numerical values are not assigned to K.

§ C_{aq} before distribution = 137 μg./ml.

‖ Volume of aqueous phase = 50 ml.; volume of organic phase = 100 ml.

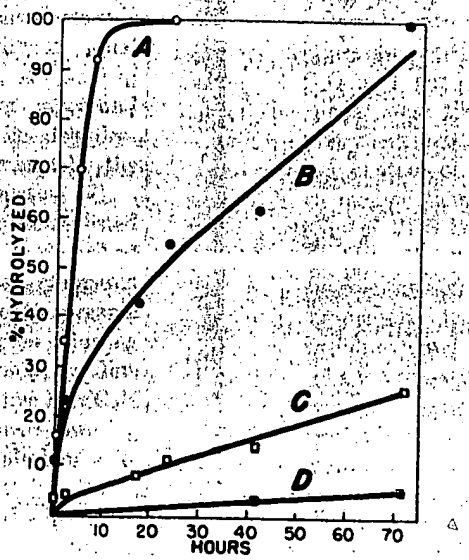

FIG. 1. Rate of hydrolysis of dehydroisoandrosterone hydrogen sulfate

A. Obtained with DHS in a homogeneous reaction mixture consisting of 6 per cent ethyl acetate and 94 per cent ether saturated with H_2O (23°). The reaction mixture was prepared by extracting DHS from a 2 N H_2SO_4 solution with ethyl acetate and diluting the resulting extract with ether saturated with water.

B. Obtained by shaking a solution of PDS in 2 N H_2SO_4 with an equal volume of ether (20°).

C. Obtained by shaking a solution of PDS made pH 1 (H_2SO_4) with an equal volume of ether (20°).

D. Obtained by shaking a solution of PDS in 2 N H_2SO_4 with an equal volume of benzene (20°).

50 ml. solutions of PDS (1.0×10^{-3} M) in (*a*) aqueous H_2SO_4 (pH 1) and (*b*) aqueous 2 N H_2SO_4 were placed in separatory funnels and were vigorously shaken for about 1 minute every 2 hours during each of 3 working days (4 times in 24 hours) with an equal volume of organic solvent. At various time intervals aliquots were withdrawn from the organic phase and the liberated dehydroisoandrosterone was determined as described above. The results are given in Fig. 1. It is clear that under the conditions of these experiments, the nature of the solvent employed and the pH of the aqueous media determine the rate of hydrolysis. Shaking a 2 N H_2SO_4 solution of PDS with benzene resulted in only 5.7 per cent hydrolysis after 72 hours (Curve *D*). At pH 1 and benzene as the organic phase, no hydrolysis took place, whereas at pH 1 and ether as the solvent 26 per cent hydrolysis was achieved (Curve *C*). In contrast, in 2 N H_2SO_4 solution with ether as the solvent, hydrolysis was quantitative (Curve *B*) in 72 hours. Using CH_2Cl_2 as solvent with 2 N H_2SO_4 as aqueous phase, only 7.5 per cent hydrolysis occurred after 48 hours. Since benzene and methylene chloride are as good extracting solvents for dehydroisoandrosterone as is ether, it is clear that hydrolysis does not proceed simply by way of constant removal of the liberated steroid.

That the hydrolysis is not catalyzed by the ether in the aqueous phase was established as follows: 22 mg. of PDS was dissolved in 100 ml. of 2 N H_2SO_4 which had previously been saturated with ether. After the solution had remained at room temperature for 9 days, it was extracted with an equal volume of benzene. Estimation of the Zimmermann positive material extracted by the benzene indicated a maximal hydrolysis of 2.4 per cent. When the residual aqueous solution was re-extracted with ether by shaking at 2 hour intervals as described above, hydrolysis similar to that illustrated in Curve *B*, Fig. 1, resulted.

Having established that the hydrolysis does not occur in the aqueous phase even in the presence of ether, the possibility suggested itself that the hydrolysis occurs in the ether phase in a manner somewhat akin to the hydrolysis in dioxane described originally by Grant and Beall (10) for estrogen sulfates and extended by Cohen and Oncley (11) to the hydrolysis of ketosteroid sulfates.[3] To investigate this possibility, the hydrolysis of DHS was carried out in homogeneous ether phase. It was found that DHS can be extracted from suitably acidified aqueous solutions using ethyl acetate as extractant. As can be seen from Line 3 in Table I, a quantitative extraction of DHS can be achieved by ethyl acetate extraction of a 2 N H_2SO_4 solution. Under these conditions, DHS in ethyl acetate hydrolyzes very slowly, but when ether saturated with water is added to the solution, rapid hydrolysis occurred (Curve *A* in Fig. 1; the specific rate constant calculated for Curve *A* is 4.6×10^{-5} sec.$^{-1}$).

An insight into the role of the acid concentration on the rate of hydrolysis with ether in the two-phase system (Fig. 1, Curves *B* and *C*) was supplied by the results of the partition experiments listed in Table I. It is evident from this table that the greater the acid content of the aqueous phase the more DHS is extracted

[3] While this manuscript was in typescript, a paper by J. McKenna and J. K. Norymberski (J. Chem. Soc., p. 3889, 1957) appeared which described solvolytic reactions of the salts of some sterol sulfates. This work complements our studies in that it represents a special case of solvolysis of organic sulfates. The results given in this paper, when considered together with ours, serve to emphasize the large inhibitory effect salt formation has upon the rate of hydrolysis.

by the organic solvent. This effect of acid is especially well demonstrated with ethyl acetate. At equal ionic strength (Systems 1 and 4, 2 and 5) an increase in the acid concentration caused a better salting out effect than did an increase in salt concentration. Since little inorganic acid is extracted by ethyl acetate (final concentration, about 10^{-4} M as determined by titration) or by ether, it is clear that the increase in the acid concentration enhances the two-phase hydrolysis with ether merely by increasing the extractability of the DHS which then solvolyzes in the organic phase in a manner described by Curve *A* in Fig. 1.

II. Procedures for the Hydrolysis of Urinary Ketosteroid Sulfates

A. A Simplified Two-Phase Hydrolysis—Based on the results reported in Section I, a simplified procedure for the hydrolysis of urinary ketosteroid sulfates which does not necessitate continuous ether extraction was developed. It consisted of bringing urine to an acid concentration of 2 N by adding the calculated amount of 50 per cent aqueous H_2SO_4 cautiously with stirring. The solution was then covered with a layer of ether equal to the volume of the urine and vigorously shaken in a cork-stoppered bottle 5 times daily for 4 days. The ether layer was then separated and worked up in the usual manner. 24 hour urine specimens were first hydrolyzed by this procedure and then the spent urines were continuously extracted with ether for 72 hours. Each ether extract was chromatographed separately by the gradient elution method (9) and the results are illustrated in Fig. 2. Although each chromatogram was run to completion (elution volume = 750 ml.) no ketosteroid was detected beyond 300 ml. of effluent and therefore the ends of the chromatograms are not depicted.

It is evident that this process affords satisfactory hydrolysis of urinary ketosteroid sulfates and that only in Experiment B was any identifiable ketosteroid found in the extract obtained by continuous extraction. In this instance only 10 per cent of the DHS present in the original urine was not hydrolyzed by the two-phase procedure involving intermittent shaking. In the other three examples the shaking procedure hydrolyzed the DHS quantitatively and in Experiments C and D liberated as well all the androsterone from its sulfate.

Another effort to assess the analytical possibilities of this procedure was directed at recovering a known sample of PDS that had been added to 1000 ml. of pregnancy urine. Previous chromatographic analysis had demonstrated that this volume of urine contained 1.9 mg. of Zimmermann reacting material in the eluates in which dehydroisoandrosterone is usually encountered. However, infrared analysis failed to establish the identity of this material. When 15 mg. of PDS (equivalent to 9.9 mg. of dehydroisoandrosterone by the Zimmermann color reaction) was added to an equal volume of this urine and the mixture hydrolyzed by Procedure A, 10.4 mg. of crystalline dehydroisoandrosterone (quantity estimated colorimetrically and identity confirmed by infrared spectrum) was obtained by chromatographic analysis.

B. Solvolysis of Urinary Ketosteroid Sulfates in Ethyl Acetate—Based on the results of the partition experiments listed in Table I and on a solvolysis study which will be reported elsewhere,[4] a simple, mild procedure was developed for the hydrolysis of urinary ketosteroid sulfates in a homogeneous ethyl acetate phase. In this process the urine can be treated in one of two ways. It may be either acidified to 2 N with H_2SO_4 (see System 3, Table I) or acidified to pH 1 and brought to a salt concentration of 20 per cent with NaCl (Table I, Solvent System 7). By shaking the urine with ethyl acetate a quantitative extraction of DHS can be achieved. The ethyl acetate extract is kept at 38° for 24 hours whereby a quantitative hydrolysis of DHS, isoandrosterone (3β-hydroxyandrostan-17-one) and androsterone sulfates is obtained. In a model experiment, an amount of PDS equivalent to 10.6 mg. of dehydroisoandrosterone was dissolved in 50 ml. of a 20 per cent NaCl solution brought to pH 1 with H_2SO_4. The solution was extracted once with an equal volume of ethyl acetate and then

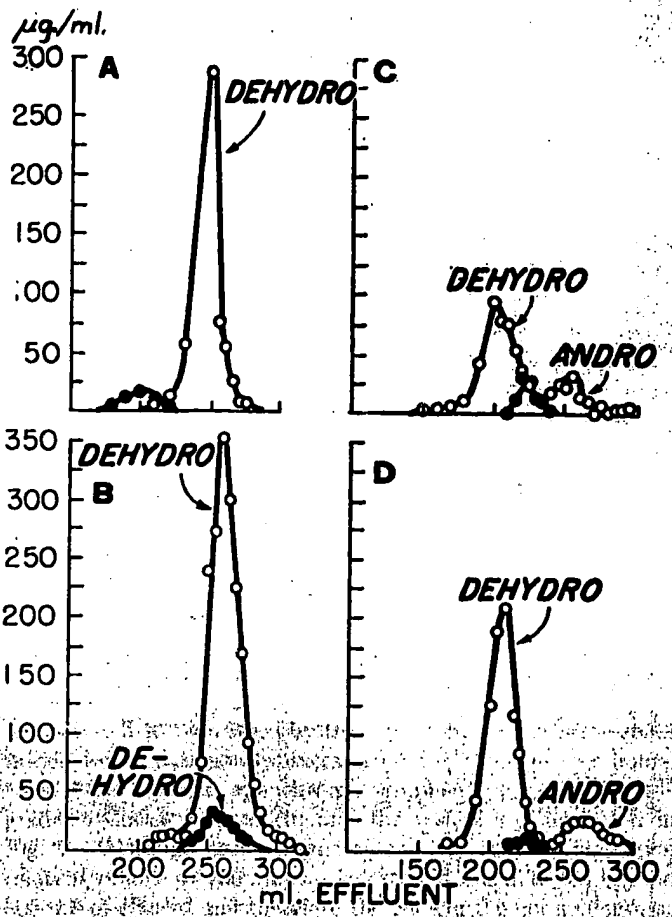

FIG. 2. Hydrolysis of urinary ketosteroid sulfates.

Chromatographic separation of urinary extracts obtained by subjecting four specimens of human urine (*A* to *D*) first to procedure A (open circles) and then to continuous extraction with ether for 72 hours (solid circles). The concentration is expressed as micrograms of dehydroisoandrosterone per ml. "Dehydro" and "andro" signify dehydroisoandrosterone and androsterone, respectively. Each was identified in appropriate eluates by infrared spectrophotometry. The contents of the other eluates were examined spectroscopically but were not identified. *A* and *B* were 24 hour urine specimens obtained from a normal man (34 years old) who regularly excretes elevated levels of dehydroisoandrosterone. *C* and *D* were two separate pools of normal men's urine.

[4] A more detailed study of the solvolysis of steroid hydrogen sulfates will be reported elsewhere. It has been shown that the solvolysis occurs in a variety of organic solvents and follows first order kinetics with respect to the sulfate. (*J. Am. Chem. Soc.*, in press.)

the organic extract left at 39° for 3 hours. At this time the ethyl acetate solution was washed with sodium bicarbonate solution and water and evaporated to dryness. Colorimetric analysis of the crystalline residue indicated 10.8 mg. of dehydroisoandrosterone. Under similar experimental conditions 5.5 mg. (expressed colorimetrically as dehydroisoandrosterone) of potassium isoandrosterone sulfate yielded 5.4 mg. isoandrosterone. Similarly, after 10 hours 6.8 mg. of potassium androsterone sulfate (expressed as dehydroisoandrosterone) yielded 6.2 mg. of androsterone. Based on these results a 24 hour solvolysis period was chosen to assure complete hydrolysis. Because of the presence of chloride ions in these experiments, about 2 per cent of the artifact, 3β-chloro-Δ⁵-androsten-17-one, was formed from DHS. Consequently, it may be advisable to replace sodium chloride with other inorganic salts. The nature of the salt, however, determines the concentration of water and mineral acid extracted into the organic phase and thereby influences the rate of solvolysis. Thus, when ammonium sulfate is substituted for sodium chloride complete hydrolysis occurs only upon raising the acid concentration in the ethyl acetate extract to about 10^{-2} M with *p*-toluene sulfonic acid.

Several recovery experiments were carried out with urine to evaluate Procedure B. To 1000 ml. of human pregnancy urine containing, as proved by previous analysis, 0.5 mg. of Zimmermann reacting but unidentifiable (infrared) material in the dehydroisoandrosterone eluates, 7.0 mg. of PDS (equivalent to 4.6 mg. of dehydroisoandrosterone) were added. Following hydrolysis by Procedure B and chromatographic analysis 5.1 mg. of dehydroisoandrosterone were recovered. Similarly when 10.6 mg. of PDS (equivalent to 7.0 mg. of the steroid) were added to 1000 ml. of normal human urine containing 0.8 mg. of identifiable dehydroisoandrosterone, 7.6 mg. were recovered.

DISCUSSION

From these studies it is clear that steroid hydrogen sulfates can be caused to solvolyze rapidly and without artifact formation merely by storing solutions of these compounds in organic solvents under appropriate conditions. Under ionizing conditions such as obtain in aqueous solutions, hydrolysis does not proceed at an appreciable rate. In all three techniques, the continuous ether extraction, Procedures A and B, hydrolysis occurs in the organic phase.

In the two-phase process using continuous ether extraction the rate of hydrolysis depends upon the rate of extraction of the hydrogen sulfate by the organic solvent and upon the rate of solvolysis in that solvent. Obviously, the first of these factors depends upon the efficiency of the extraction apparatus and the partition coefficient of the sulfate in the solvent system used. In Procedure A, where a noncontinuous extraction system was used, the rate of hydrolysis necessarily depends upon the frequency of shaking. For example, when the experiment, whose course is illustrated by Curve *C* in Fig. 1, was repeated with continuous mechanical shaking,[5] 20 per cent hydrolysis occurred within 15 hours. The inefficacy of benzene or methylene chloride as solvents in the two-phase procedure is primarily due to the low distribution coefficients of the hydrogen sulfates in these solvents (Table I).

In Procedure B advantage was taken of the high extractability of the steroid hydrogen sulfates by ethyl acetate and the relatively rapid solvolysis that occurs in this solvent under the conditions described. In this method, the steroid hydrogen sulfate must be kept in solution for should it become insoluble, as might occur during concentration of the solution, it will escape hydrolysis.

The dioxane procedures of Grant and Beall (10) and of Cohen and Oneson (11) are likewise examples of solvolysis in an organic phase. They differ from the procedures described here in that both techniques require that the sulfate be isolated in reasonably pure form, free of water and interfering substances. Prior to hydrolysis in the dioxane-trichloroacetic acid mixtures, the latter procedure (11) requires extraction of the sulfate from urine with butanol, washing the butanol extract to remove interfering substances and evaporation of the solvent *in vacuo*.

The procedures described in this paper afford a quantitative hydrolysis of urinary steroid sulfates by simple and convenient techniques. The necessity of employing continuous extraction apparatus is eliminated, thereby facilitating the routine handling of urine specimens, regardless of the number or volume. Extraction by the conventional Kutscher-Steudel apparatus is often attended by practical difficulties such as thick emulsions, carrying over of acidified urine into the warm organic extract, and inefficient extraction due to mechanical causes. Such difficulties often lead to incomplete extraction and to misinterpretation of the nature of the urinary conjugates. Procedures A and B obviate the need for elevated temperatures and high acidities. The concentration of acid in the solvolytic procedures is about 10^{-4}–10^{-2} M and this feature may have important bearing upon the recovery of acid sensitive steroids such as the corticoids.

The use of sulfatase for the hydrolysis of urinary steroid sulfates offers no particular advantage. In fact, since the mammalian (12) and molluscan (13) enzymes are specific only for 3β-sulfates of the 5α or Δ⁵ series of steroids they are not generally applicable. Procedures A and B both provide complete hydrolysis of the sulfates of dehydroisoandrosterone, androsterone and isoandrosterone under the conditions set forth. Thus both the saturated and unsaturated sulfates are hydrolyzed in the organic phase in Procedures A and B as proved by the model experiments and by the isolation of dehydroisoandrosterone and androsteron from two separate pools of urine.

Although these procedures have been successfully applied to urine, it must be emphasized that they do not necessarily represent the best solvolytic procedures possible. As they are constituted, they involve only a few of a great many variations that can be devised using the principles outlined. It may be possible to develop superior methods of hydrolysis merely by selecting solvents which extract the sulfates more efficiently and which will cause them to solvolyze more rapidly.

Although the observations described in this paper pertain only to C_{19}-steroid hydrogen sulfates, the results are undoubtedly applicable to other classes of organic sulfates. On the basis of the kinetic studies to be reported,[4] it has been possible to propose a general mechanism which indicates that solvolysis of any organic hydrogen sulfate will occur when and if that compound is dissolved in a suitable nonpolar medium. Thus a method is provided for the mild hydrolysis of other naturally occurring sulfates and this may aid significantly in the isolation of hitherto inac-

[5] With continuous mixing under equilibrium conditions, the first order specific rate of hydrolysis for a two-phase process is given by $K_s K_p/1 + K_p$, where K_s is the specific rate of solvolysis in the organic phase and K_p the partition coefficient of the sulfate between the two phases.

cessible compounds. On the other hand, in some instances the facile splitting of sulfate groups may be a disadvantage. For example, the observations reported here may have special relevance for procedures used in the isolation of lipid sulfates or sulfatides. Any technique which involves extraction of the lipid hydrogen sulfates or even the salts of the lipid sulfates[9] into nonpolar organic solvents may result in the loss of some or all of the sulfate groups.

SUMMARY

The continuous ether procedure (2) for the hydrolysis of steroid sulfates has been found to proceed in two stages: first, extraction of the steroid hydrogen sulfate into the ether phase and then, its solvolysis in the organic solvent. The general principles involved in this mild solvolytic splitting of organic hydrogen sulfates have been discussed and illustrated with steroid sulfates and with urinary samples. Two new procedures for the hydrolysis of urinary steroid sulfates have been presented. Both methods are based upon the observation that steroid hydrogen sulfates are rapidly solvolyzed at low temperatures when dissolved in an appropriate nonpolar medium.

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Thanks

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Preparation f 16α-Hydroxypregn nol n -3-sulfate and a Study of Its An malous Behavior During Hydrolysis

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ABSTRACT. Nonlabeled and high specific activity 7-^{3}H-16α-hydroxypregnenolone-3-sulfate were prepared by microbiological hydroxylation of pregnenolone-3-sulfate and 7-^{3}H-pregnenolone sulfate with *Streptomyces roseochromogenes* in 58 and 68% yield, respectively. Solvolysis, with perchloric acid in tetrahydrofuran, of the 16α-hydroxypregnenolone sulfate in the pure form and also when added to urine resulted in the liberation of 16α-hydroxypregnenolone and a second steroid identified as 16-dehydropregneno lone. Following Glusulase hydrolysis of 16α-hydroxypregnenolone sulfate the nonconjugated steroid and 16α-hydroxyprogesterone were identified. This transformation occurs only when the pure conjugated steroid is hydrolyzed with Glusulase and does not take place when it is added to the urine prior to hydrolysis. (*Endocrinology* **84**: 1230, 1969)

THE ROLE of 16α-hydroxysteroids, such as 16α-hydroxydehydroepiandrosterone and its sulfate,[1] in the formation of estriol in the human fetoplacental unit has by now been extensively studied. Other 16α-hydroxylated steroids, such as 16α-hydroxypregnenolone, have been isolated from the human fetal liver following the injection of labeled pregnenolone into the umbilical vein at midpregnancy (1), from the urine of normal infants and children (2), from the urine of premature infants (3) and from the urine of infants with the 3β-hydroxysteroid dehydrogenase deficiency type of adrenogenital syndrome (4). In order to study the metabolism of 16α-hydroxypregnenolone-3-sulfate we prepared the labeled steroid sulfate and during the course of proving its structure a number of anomalous reactions were observed which led us to investigate the stability of this sulfate during hydrolysis. This paper describes the synthesis of labeled 16α-hydroxypregnenolone-3-sulfate and its transformation to 16-dehydropregnenolone during acid solvolysis. In addition, it was found that 16α-hydroxypregnenolone and its 3-sulfate were converted to 16α-hydroxyprogesterone during Glusulase hydrolysis.

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[1] The following trivial names are used: 16α-hydroxypregnenolone-3-sulfate = 16α-hydroxy-20-oxopregn-5-ene-3β-yl sulfate; pregnenolone sulfate = 20-oxopregn-5-ene-3β-yl sulfate; pregnenolone = 3β-hydroxypregn-5-en-20-one; 16α-hydroxypregnenolone = 3β,16α-dihydroxypregn-5-en-20-one; 16-dehydropregnenolone = 3β-hydroxypregn-5,16-dien-20-one; 16α-hydroxyprogesterone = 16α-hydroxypregn-4-en-20-one; 16α-hydroxydehydroepiandrosterone-3-sulfate = 16α-hydroxy-17-oxoandrost-5-ne-3β-yl-sulfate; 16-dehydroandrosten-3α-ol = 5α-androst-16-en-3α-ol; 16-dehydropregnan-3β-ol = 3β-hydroxy-5α-pregn-16-en-20-one; 16-d hydropregnan-3α-ol = 3α-hydroxy-5β-pregn-16-en-20-on .

Materials and Methods

All solvents used were distilled prior to use and were peroxide free. Tetrahydrofuran was refluxed for at least 2 hr with potassium hydroxide prior to distillation. Melting points were determined on a Kofler block and are corrected. Infrared spectra (KBr) were obtained with a Perkin-Elmer infrared spectrometer (Model 221). Radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 4322). The samples were first dissolved in 2 ml of methanol followed by the addition of 10 ml of toluene phosphor solution containing 0.3% (w/v) of 2,5-diphenyloxazole and 0.01% of 1,4-bis-(5-phenyloxazolyl)-benzene. The efficiency of counting tritium under these conditions was 16%.

Purification of steroids isolated in these studies was accomplished by the use of paper partition chromatography with the solv nt systems given in Table 1. Steroids were eluted

TABLE 1. Solvent systems used for paper chromatography

System	Solvents
A	Isopropylether:*t*-butanol:ammonium hydroxide (1.5M) (3:5:8)
B	Toluene:*n*-butanol:ammonium hydroxide:water (10:10:2:18)
C	Methylcyclohexane:ethyl acetate:*n*-butanol:methanol:ammonium hydroxid (1M) (25:40:8:20:30)
D	2,2,4-Trimethylpentane:toluene:methanol:water (3:5:4:1)
E	Benzene:methanol:water (2:1:1)
F	Toluene:propylene glycol
G	2,2,4-Trimethylpentane:*t*-butanol:methanol:water (50:10:35:5)
H	Skellysolve C:benzene:methanol:water (10:5:8:2)
I	*n*-Butanol:benzene:methanol:water (10:1:3:3)

from the paper chromatograms with methanol and in some instances were further purified by chromatography on small silica gel or alumina columns as described previously (5). Steroid sulfates were prepared by the procedure described by Fieser (6) and were converted to the ammonium salts by dissolving the pyridinium salts in 7M ammonium hydroxide followed by extraction with *n*-butanol. Steroid sulfates were 16α-hydroxylated by incubation with a strain of *Streptomyces roseochromogenes* (ATCC 3347), supplied through the courtesy of Dr. C. Vézina, Ayerst Laboratories, Montreal. The procedure used for the incubation was the same as the one previously described for the synthesis of (7-^{3}H)-16α-hydroxyprogesterone (5).

Steroid sulfates were detected on paper chromatograms either with methylene blue reagent (8) or by scanning the paper in a Packard (Model 7200) Radiochromatogram Scanner. Steroids were identified by either the classic methods, such as melting points, mixed melting points and infrared spectra, or by the reverse isotope dilution technique. Radiochemical purity of a steroid was established when constant specific activities were obtained both in the unaltered steroid and in a derivative.

Results

Preparation of 16α-hydroxypregnenolone-3-sulfate. A total of 245 mg of freshly prepared pregnenolone sulfate was incubated with *Streptomyces roseochromogenes* for three days as previously described (5). After incubation, the medium was extracted two times with twice the volume of *n*-butanol; the butanol extract was washed with water and evaporated to dryness under vacuum. The residue was partitioned between ether and water and the ther extract discarded. The aqueous phase was re-extracted with *n*-butanol as described above and the butanol layer was evaporated to yield 8.6 g of a dark brown residue. This residue was dissolved in methanol and 100 mg of activated charcoal was added and the mixture warmed on a steam bath and then filtered. The filtrate was evaporated under vacuum to yield 3.8 g of a light straw colored residue. This residue was purified by paper chromatography on Whatman 3 MM paper using system A (20 papers), B and C consecutively. The product eluted from the final chromatogram weighed 280 mg and, after crystallization from methanol, ethanol and methanol-hexane, 145 mg of crystals was obtained (58% yield).

This material had a mp of 214–217 C and the sulfur analysis for $C_{21}H_{35}O_6NS$ (NH_4 salt) was found to be 7.38%, in agreement with the calculated value of 7.46%. Its infrared spectrum (Fig. 1) had absorption bands at 1691 cm^{-1} (20-ketone), 1635 cm^{-1} (C=C), and 1220–1255 cm^{-1} (3-sulfate).

One mg of the crystalline material was solvolyzed in tetrahydrofuran (100 ml) and perchloric acid (0.11 ml of 60% perchloric acid/100 ml of solution at 37 C for 17 hr) in accordance with the procedure of Jacobsohn and Lieberman (9). The solvolyzed product was purified by paper chromatography in systems D and E and in both these systems it had the mobility of 16α-hydroxypregnenolone. Then the material was chromatographed on a small silica gel column and elution with 9% ethanol in benzene yielded a product which was recrystallized from methanol and ethanol-

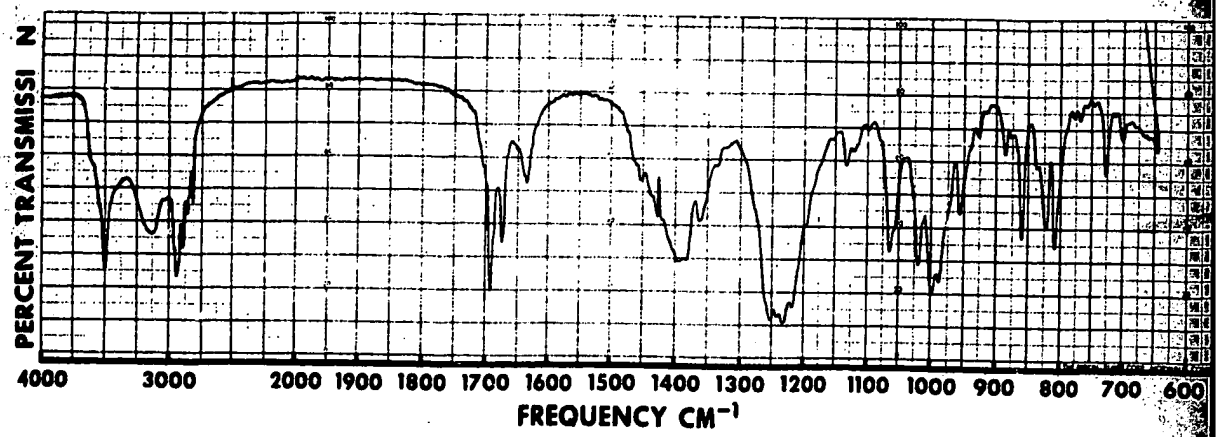

FIG. 1. Infrared spectrum (KBr) of 16α-hydroxypregnenolone-3-sulfate.

hexane. The crystals had a mp of 230–234 C, a mixed mp of 230–233 C with authentic 16α-hydroxypregnenolone (mp 230–232 C), and an infrared spectrum identical with that of 16α-hydroxypregnenolone.

Preparation of 7-³H-16α-hydroxypregnenolone-3-sulfate. Four mCi of 7-³H-pregnenolone (New England Nuclear Corp.) having a specific activity of 16.0 Ci/mmole was converted to the ³H-pregnenolone-3-sulfate by reacting it with pyridine and chlorosulfonic acid as described above. The pyridinium salt was converted to the ammonium salt by reacting it with 10 volumes of 7M ammonium hydroxide and the reaction mixture was extracted three times with *n*-butanol, and the butanol layer was washed with water and evaporated to dryness. The residue weighing 1.7 mg was chromatographed on Whatman 3 MM paper in system C for five hours. Two main radioactive products were observed, one migrating with the solvent front and a polar compound corresponding in mobility to pregnenolone sulfate. This latter material was eluted and the eluate filtered and evaporated to dryness. The residue was next chromatographed on system A for 15 hours and then in system C for 18 hours. A single radioactive compound was observed in both systems corresponding in m bility to pregnenol ne sulfate. The material luted from the sec nd chromatogram contained 2.4 × 10^9 cpm and it was then incubated with *Streptomyces roseochromogenes* as described above. The incubation medium was extracted with *n*-butanol, and the product obtained was purified as described, except that the treatment with charcoal was omitted. A single major radioactive peak corresponding in mobility to 16α-hydroxypregnenolone sulfate was obtained after chromatography in system C. The material eluted from this zone contained 1.6×10^6 cpm, which represented a yield of 66%. This material was rechromatographed on paper using system A, and after elution (1.4×10^6 cpm) was mixed with 564 μg of carrier 16α-hydroxypregnenolone-3-sulfate (NH_4 salt). The mixture was chromatographed in systems A and C and a single peak of radioactive material corresponding in mobility to 16α-hydroxypregnenolone-3-sulfate was obtained in both systems. The radiochemical purity of the eluted material was established by the use of isotope dilution technique as shown in Table 2. After constant specific activity was obtained from the crystallization of the sulfate, a second aliquot containing 2.9×10^4 cpm was solvolyzed and the nonconjugated product was chromatographed on paper in systems E and D. The radioactive material corresponding in mobility to 16α-hydroxypregnenolone was eluted and 1.18×10^4 cpm of this material was mixed with 18.6 mg of carrier and the mixture crystallized to constant specific activity as shown in Table 2.

TABLE 2. Proof of radiochemical purity f (7-³H)-16α-hydroxypregnenol ne-3-sulfate

Crystallization	Specific activity (cpm/mg)	
	16α-Hydroxy-pregnenolone-3-sulfate*	16α-Hydroxy-pregneno-lone†
1	1410	670
2	1490	680
3	1390	670
Calculated	1460	630

* An aliquot containing 2.9×10^4 cpm was mixed with 19.1 mg of carrier 16α-hydroxypregnenolone-3-sulfate (NH_4 salt) prior to crystallization and the calculated specific activity was based on these values.

† A total of 1.84×10^4 cpm of the solvolyzed product was mixed with 18.6 mg of carrier 16α-hydroxypregnenolone prior to crystallization.

TABLE 3. Proof of radiochemical purity f 16α-hydroxypregnen lon

Crystallization	Specific activity (cpm/mg)	
	16α-Hydroxy-pregneno-lone	16α-Hydroxy-pregnenolone-diacetate†
1	2350	2390
2	2260	2380
3	2260	
Calculated*	2520	2260

* A total of 5.5×10^4 cpm was mixed with 22 mg of carrier 16α-hydroxypregnenolone prior to crystallization.

† The 3rd crystals were acetylated and the specific activities have been corrected for changes in molecular weight.

Formation of 16-dehydropregnenolone during hydrolysis. Following solvolysis of ³H-16α-hydroxypregnenolone sulfate, a small amount of labeled material less polar than 16α-hydroxypregnenolone was noted on the paper chromatograms. In order to identify this material, the following experiment was performed. A total of 6.05 mg of 7-³H-16α-hydroxypregnenolone sulfate containing 7.8×10^4 cpm was solvolyzed with tetrahydrofuran and perchloric acid at 37 C for 17 hours. After neutralizing the perchloric acid with NH_4OH, the tetrahydrofuran was evaporated and a neutral extract was prepared. The residue thus obtained weighed 7.63 mg and contained 7.4×10^4 cpm. It was chromatographed on paper in system E and two zones of radioactive material were observed. The more polar material contained 74% of the original radioactivity (5.4×10^4 cpm) and it was identified as 16α-hydroxypregnenolone as described above. The less polar UV positive material weighed 1.1 mg and contained 6.6×10^3 cpm and represented 9.4% of the original radioactive material. It was further purified by paper chromatography in system G. The material eluted from the paper was chromatographed on a 1 g silica gel column and elution with 3% methanol in benzene yielded 400 μg of a product which after crystallization from acetone-ligroin B gave 110 μg of a crystalline material having a mp of 211–213 C and a mixed mp of 213–214 C with authentic 16-dehydropregnenolone (mp 212–213 C). Its IR spectrum was identical with that of 16-dehydropregnenolone. In order to demonstrate that the dehydration was caused by the acid, the following experiment was done.

A total of 8.9×10^4 cpm of ³H-16α-hydroxypregnenolone-3-sulfate was refluxed for 4 1/2 hours with 100 ml of freshly distilled tetrahydrofuran in the absence of acid. Then the tetrahydrofuran was evaporated to dryness and the residue was processed as described above. After chromatography only one main area of radioactive material was obtained corresponding in mobility to 16α-hydroxypregnenolone. This material was identified as 16α-hydroxypregnenolone by crystallization to constant specific activity after addition of carrier, as shown in Table 3. It is of interest to note that over 90% of ³H-16α-hydroxypregnenolone sulfate was hydrolyzed to ³H-16α-hydroxypregnenolone by this procedure. When ³H-16α-hydroxypregnenolone-3-sulfate was added to nonpregnancy urine and a tetrahydrofuran extract, prepared in the absence of acid, was refluxed as described above, no solvolysis of the conjugate was observed and no 16-dehydropregnenolone was detected. When the tetrahydrofuran extract was refluxed in the presence of perchloric acid, solvolysis occurred and sizable

TABLE 4. Radiochemical purity of 16-dehydropregnen 1 ne isolated after solvolysis of n npregnant and pregnancy urine containing trace amounts of ^{3}H-16α-hydroxypregnenol n -3-sulfate

Crystallization	Specific activity (cpm/mg)			
	Nonpregnancy urine		Pregnancy urine	
	16-Dehydro-pregnenolone	16-Dehydro-pregnenolone acetate	16-Dehydro-pregnenolone	16-Dehydro-pregnenolone acetate
1	1270	1390	1230	1260
2	1330	1390	1290	1250
Calculated	1280*	1330†	1200‡	1290†

* The calculated specific activity was obtained by adding 11 mg of carrier 16-dehydropregnenolone to 1.4×10^4 cpm prior to crystallization of the mixture.
† The 2nd crystals were acetylated and the specific activities have been corrected for changes in molecular weight.
‡ The calculated specific activity was obtained by adding 10 mg of carrier 16-dehydropregnenolon to 1.2×10^4 cpm prior to crystallization of the mixture.

amounts of 16-dehydropregnenolone were formed.

The next question that arose was whether the dehydration could occur with the nonconjugated steroid and the following experiment was performed to clarify this issue. A total of 1.6×10^6 cpm of ^{3}H-16α-hydroxypregnenolone was subjected to the solvolytic procedure with tetrahydrofuran and perchloric acid at 37 C for 17 hours. The neutral extract was prepared and processed as described earlier. Two areas of radioactive material were observed following paper chromatography of the extract in system E. The major product corresponded in mobility to 16α-hydroxypregnenolone; the less polar material contained approximately 14% of the original radioactivity and was identified as 16-dehydropregnenolone by the isotcpe dilution technique after addition of carrier 16-dehydropregnenolone. These results indicate that dehydration also occurs with the nonconjugated steroid.

Solvolysis of ^{3}H-16α-hydroxypregnenolone sulfate in the urine of the pregnant and nonpregnant female. In order to ascertain whether dehydration of 16α-hydroxypregnenolone-3-sulfate can occur in urine, the following set of experiments was performed. To separate 100 ml portions of nonpregnant and late pregnancy urine was added 1.8×10^5 cpm of ^{3}H-16α-hydroxypregnenolone sulfate. The urines were extracted and solvolyzed as described by Jacobsohn and Lieberman (9), and the neutral extract from each experiment was chromatographed on paper using system E. Two areas of radioactive material were again observed. From the extract of the nonpregnancy urine, the material corresponding in mobility to 16α-hydroxypregnenolone contained 76% of the radioactivity and the less polar area, corresponding to 16-dehydropregnenolone, contained 16% of the original radioactivity. The corresponding values for the extracts from the pregnancy urin were 71 and 12%, respectively. In both experiments the material corresponding in mobility to 16-dehydropregnenolone was eluted and rechromatographed in system G and was then purified by chromatography on a 1 g alumina column. The material eluted with 0.5 to 1% ethanol in benzene was identified as 16-dehydropregnenolone by isotopic dilution analysis in the manner shown in Table 4. These results clearly indicate that dehydration during solvolysis also takes place in both nonpregnancy and pregnancy urines.

Hydrolysis of ^{3}H-16α-hydroxypregnenolone sulfate with Glusulase. A second method for hydrolysis of ^{3}H-16α-hydroxypregnenolone-3-sulfate consisted of incubating the conju-

gate with Glusulase (Endo Laboratories, Garden City, N.Y.) according to the procedure described by Ruse and Solomon (5). In one such experiment, 5.7×10^5 cpm of ^{3}H-16α-hydroxypregnenolone-3-sulfate was dissolved in 100 ml of distilled water and 5 ml of acetate buffer (pH 5.2) as well as 0.5 ml of Glusulase (50,000 units of glucuronidase, 25,000 units of sulfatase) were added. The mixture was incubated for four days at 37 C. It was found that 62% of the conjugate was hydrolyzed by this treatment and the resulting neutral extract was chromatographed in system E. Four areas of radioactive material were observed (zones 1 to 4 in decreasing polarity). The least polar material, zone 4, accounted for 49% of the radioactivity in the hydrolyzed fraction, while zones 1, 2 and 3 contained 11, 14 and 3% of the radioactivity, respectively. The material in zone 2 corresponded in mobility to 16α-hydroxypregnenolone, while the materials in zones 1 and 3 have not as yet been identified. The radioactive material in zone 4 was further purified by chromatography in systems F and I. In all the three chromatographic systems used the material had the mobility of 16α-hydroxyprogesterone. Its identity was established by the isotope dilution technique as described above.

Conversion of 16α-hydroxypregnenolone to 16α-hydroxyprogesterone by Glusulase. The previous experiment indicated the presence of a Δ^5-3β-hydroxysteroid dehydrogenase/isomerase in Glusulase and it was of interest therefore to determine whether the 16α-hydroxypregnenolone, rather than its sulfate, could be converted to 16α-hydroxyprogesterone and such an experiment is described below.

Two mg of ^{3}H-16α-hydroxypregnenolone, containing 1.9×10^6 cpm, was incubated with Glusulase for four days as described above. The neutral extract, containing 1.6×10^6 cpm, was chromatographed on paper in system E. Four main areas of radioactiv material were observed on the paper chromatograms and approximately 35% of the original radioactivity was present in the area corresponding in mobility to 16α-hydroxypregnenolone, and was not further processed. A UV positive material which was less polar than 16α-hydroxypregnenolone was also observed. This material contained 39% of the original radioactivity and was further purified by chromatography using systems G and F. The compound eluted from the last chromatogram was further purified by chromatography on a 1 g alumina column. Elution with 1.5% ethanol in benzene gave 950 μg of a white residue which on crystallization yielded a product whose infrared spectrum was identical with that of 16α-hydroxy progesterone and had a mp of 218–222 C and a mixed mp of 217–222 C, with authentic 16α-hydroxyprogesterone (mp 217–222 C). In contrast to the above findings, when trace amounts of ^{3}H-16α-hydroxypregnenolone-3-sulfate were added to pregnancy and nonpregnancy urines and the Glusulase hydrolysis was performed, the formation of 16α-hydroxyprogesterone or 16-dehydropregnenolone was not observed.

When bacterial β-glucuronidase (Sigma Type 1) was used to hydrolyze ^{3}H-16α-hydroxypregnenolone-3-sulfate the formation of 16α-hydroxyprogesterone was not observed and only 15% of the radioactive material was hydrolyzed to a product which has not as yet been identified.

Discussion

This report describes a simple method of preparing tritium-labeled and nonlabeled 16α-hydroxypregnenolone-3-sulfate by microbiological hydroxylation of pregnenolone sulfate with a strain of *Streptomyces roseochromogenes*. A similar method has been described for 16α-hydroxylation of dehydroepiandrosterone-3-sulfate (7). The structure of 16α-hydroxypregnenolone-3-sulfate was determined from its infrared spectrum, sulfur analysis, and from its conversion to 16α-hydroxypregnenolone after solvolysis.

During solvolysis with perchloric acid in

tetrahydrofuran, a less polar UV absorbing material was formed which was identified as 16-dehydropregnenolone. This observation is significant since steroids such as 16-dehydroandrostan-3α-ol (12–15), 16-dehydropregnan-3β-ol (16) and 16-dehydropregnan-3α-ol (17) have been isolated from urine after subjecting the urinary conjugate to various forms of hydrolysis. The results indicate that dehydration at position 16 occurs with 16α-hydroxypregnenolone-3-sulfate as well as with 16α-hydroxypregnenolone. These experiments were done with pure steroids and the question arose as to whether this dehydration would occur in a medium such as urine. When 16α-hydroxypregnenolone sulfate was added to pregnancy and nonpregnancy urine and the solvolysis repeated, the formation of 16-dehydropregnenolone was again observed. These results indicate the need to apply some caution in assigning an origin to the 16-dehydrosteroids isolated from urine which has been subjected to solvolysis by the procedure described herein.

When 16α-hydroxypregnenolone-3-sulfate was refluxed with tetrahydrofuran for four hours, in the absence of any acid, 90% of the conjugate was solvolyzed to 16α-hydroxypregnenolone but no 16-dehydro or any other steroid could be detected. The results obtained from solvolysis in the presence and absence of acid indicate that dehydration is an acid catalyzed reaction. A number of similar acid catalyzed dehydrations have been previously described and are reviewed by Dorfman and Ungar (10). Solvolysis of steroid sulfates in tetrahydrofuran in the absence of acid has been previously described (11).

This unusual behavior of 16α-hydroxypregnenolone-3-sulfate led us to investigate the hydrolysis of this conjugate with enzyme preparations. Glusulase, which contains both sulfatase and β-glucuronidase, has been used very frequently to hydrolyze urinary conjugates and w attempted to hydrolyze ^{3}H-16α-hydroxypregnenolone-3-sulfate with this enzyme preparation. Following such an experiment it was foun that 49% of the hydrolyzed radioactiv material was in the form of 16α-hydroxy progesterone, while only 14% was recov ered as 16α-hydroxypregnenolone. Thes results point to the presence of a Δ^5-3β hydroxysteroid dehydrogenase in the Glu sulase preparation used. When the steroi conjugate was added to both pregnancy and nonpregnancy urine and the hydrolysis with Glusulase was repeated, no 16α-hydroxyprogesterone could be detected and the only product found was 16α-hydroxy-pregnenolone. Thus, it appears that there is a factor present in the urine which inhibits the Δ^5-3β-hydroxysteroid dehydrogenase activity present in the Glusulase preparation. There is good evidence to indicate that the 16-dehydrosteroids are naturally occurring compounds. A review of the literature concerning the isolation of 16-dehydrosteroids from urine indicates that 3α-hydroxy-5α-androst-16-ene (12–14) was isolated under conditions where the dehydration in ring D described herein was not likely to occur. The same consideration holds for the isolation of urinary 3α-hydroxy-5β-pregn-16-en-20-one (18). In addition, it has been demonstrated that a number of labeled precursors can be converted to 16-dehydrosteroids by animal testes and adrenals incubated *in vitro* (19–23). The isolation of 16-dehydrosteroids in these *in vitro* studies did not involve the use of hydrolytic procedures.

In summary, the data presented indicate that it is possible to form 16-dehydrosteroids when extracts containing 16α-hydroxypregnenolone and its sulfate are submitted to acid catalyzed hydrolysis. Glusulase hydrolysis leads to the transformation of 16α-hydroxypregnenolone and its sulfate to 16α-hydroxyprogesterone when pure steroids are substrates but this conversion was not observed when the steroids were added to urine. These findings point to a need for caution when the hydrolysis of steroid conjugates is used as the only means for identifying such compounds.

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Thank you

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DETERMINATION OF UNCONJUGATED AND SULFATED NEUTRAL STEROIDS IN HUMAN FETAL BLOOD OF EARLY AND MID-PREGNANCY

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ABSTRACT

Pooled and individual plasma samples from human fetuses of 10-20 weeks' gestational age were collected from the fetal and placental sides after dissection of the umbilical cord. Fractions of unconjugated and mono- and disulfates of neutral steroids were separated and the compounds in these fractions were identified and estimated, using gas-liquid chromatography and gas chromatography - mass spectrometry. Only progesterone was found as an unconjugated steroid exceeding the limit of detection (about 1 μg/100 ml of blood plasma). 16α-Hydroxydehydroepiandrosterone, dehydroepiandrosterone, pregnenolone and 3β,17β--dihydroxy-5-androsten-16-one were the main compounds in the monosulfate fraction and 5-androstene-3β,17α-diol among the disulfates. 16α-Hydroxydehydroepiandrosterone, 16α-hydroxypregnenolone and 3β,17α-dihydroxy-5-pregnen-20-one monosulfates were present in higher concentrations in the blood flowing to the placenta than in the blood returning from it.

INTRODUCTION

An extensive metabolism of neutral steroids, many of which serve as estrogen precursors, has been demonstrated to take place in the feto-placental unit (see e. g. 1, 2). In investigations from this laboratory, endogenous neutral steroid mono- and disulfates have been determined in some fetal tissues (3, 4) and considerable differences were observed in the amounts of these conjugates in term amniotic fluid as compared to those in the amniotic fluid collected in early and mid-pregnancy (5, 6). There are several reports on neutral steroids in cord blood of newborns

(7-21) but there seem to be no data on the endogenous neutral steroids in the blood of fetuses at earlier stages of gestation. In this study, unconjugated as well as mono- and disulfates of neutral steroids were identified and determined in fetal blood collected in early and mid-pregnancy.

MATERIAL AND METHODS

Fetal blood was obtained during interruption of pregnancy (of 10-20 weeks' duration) for socio-medical reasons. The fetus was delivered by abdominal hysterotomy, the umbilical cord was dissected and blood flowing from the fetal and from the placental side was collected in separate heparinized tubes. The plasma was separated by centrifugation and the samples were stored at -20°C until analyzed. Due to the small amounts of blood obtained, most analyses were performed from pooled samples.

Solvents and reference compounds were as described previously (see e. g. 3).

Trivial and systematic names of steroids. Dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; 16α-hydroxydehydroepiandrosterone, 3β,16α-dihydroxy-5-androsten-17-one; pregnenolone, 3β-hydroxy-5-pregnen-20-one; 16α-hydroxypregnenolone, 3β,16α--dihydroxy-5-pregnen-20-one, progesterone, 4-pregnene-3,20-dione.

Procedure. The plasma samples (2.4 - 5 ml) were processed essentially as described previously for adult human plasma (22). After extraction, the samples were chromatographed on Sephadex LH-20 and fractions of unconjugated and mono- and disulfates of neutral steroids were obtained. The sulfate conjugates were cleaved by solvolysis, and the unconjugated steroid fraction was purified by solvent partition between ethyl acetate and 0.1 N sodium hydroxide. The compounds in the three fractions were further fractionated according to polarity on a 3-g column of silicic acid (23). Finally, the silicic acid fractions were purified on a 200-mg silicic acid column (22).

The compounds in the fractions derived from the unconjugated steroids were analysed after the formation of O-methyl oxime trimethyl silyl (MO-TMS) ether derivatives (see e. g. 24) and those from the sulfated fractions after the formation of trimethyl silyl (TMS) ethers (see e. g. 3). In quantitative analyses, a known amount of stigmasterol (9 μg) was added to the samples prior to the formation of the derivatives and the measurements were performed as described previously (25).

Gas-liquid chromatography (GLC) was performed on 3 % QF-1 and 2.2 % SE-30 columns, using a flame ionization detector as described previously (25).

Gas chromatography-mass spectrometry (GC-MS) was carried out with an LKB 9000 instrument (LKB-Produkter AB, Stockholm--Bromma, Sweden), using QF-1 and SE-30 columns. The energy of the bombarding electrons was 70 eV and the ionizing current 60 μA.

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A compound was regarded as identified when the relative retention times (RRT) of its TMS or MO-TMS derivatives and the mass spectra of these derivatives were the same as those of an appropriate reference compound.

RESULTS

Table 1 summarizes the steroids identified in the unconjugated and mono- and disulfate fractions of fetal blood plasma. The mass spectra of these compounds have been presented earlier (3, 25-29) and will not be discussed again here. Fig. 1 shows GLC analyses of two silicic acid fractions of the monosulfates and Fig. 2 a GLC analysis of the disulfate fraction.

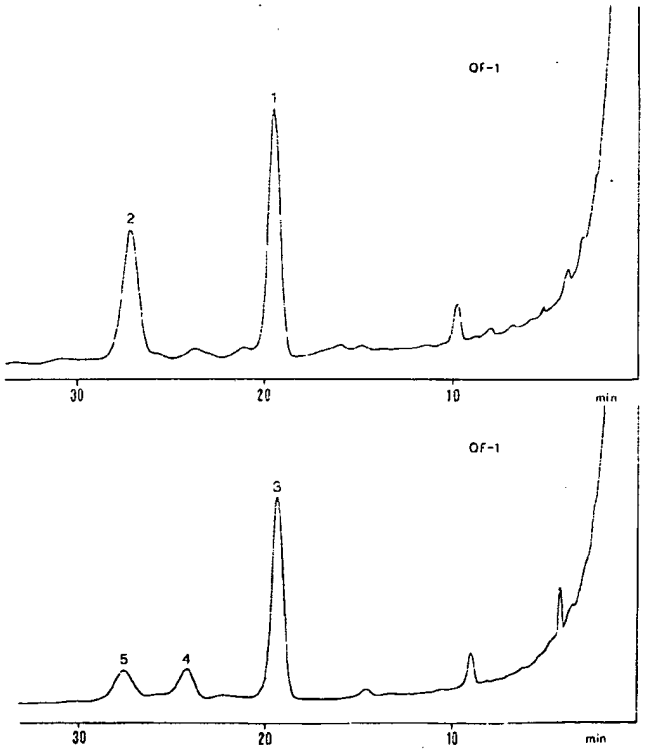

Figure 1. GLC analyses of the TMS ethers of neutral steroids in the monosulfate fraction of fetal blood plasma. Upper curve: Fraction eluted from a 3-g column of silicic acid with 20 ml of 27 % ethyl acetate in benzene; lower curve: Fraction eluted with 20 ml of ethyl acetate. 3 % QF-1 column, 215°. The numbers refer to the TMS ether derivatives of 1 = dehydroepiandrosterone, 2 = pregnenolone, 3 = 16α-hydroxydehydroepiandrosterone, 4 = 3β,17β-dihydroxy-5-androsten-16-one, 5 = 16α-hydroxypregnenolone.

Table 1. Relative retention times (5α-cholestane = 1.00) of the trimethyl silyl derivatives of neutral steroids identified in human fetal plasma. Chromatographic conditions: 3 % QF-1, 215°; 2.2 % SE-30, 225°.

Identification	QF-1		SE-30	
	Compound from plasma	Reference compound	Compound from plasma	Reference compound
MONOSULFATES				
Dehydroepiandrosterone	1.31	1.30	0.47	0.47
5-Androstene-3β, 17α-diol	0.48	0.48	0.53	0.53
5-Androstene-3β, 17β-diol	0.58	0.57	0.61	0.61
3β, 16α-Dihydroxy-5-androsten-17-one	1.42	1.43	0.84	0.85
3β, 17β-Dihydroxy-5-androsten-16-one	1.78 - 1.81	1.80	0.93	0.94
Pregnenolone	1.80 - 1.83	1.81	0.78	0.78
5-Pregnene-3β, 20α-diol	1.06	1.06	1.12 - 1.14[x]	1.14
3β, 16α-Dihydroxy-5-pregnen-20-one	1.97 - 1.99	1.97	1.27 - 1.29	1.26
3β, 17α-Dihydroxy-5-pregnen-20-one	1.97 - 2.00	1.98	1.12 - 1.14[x]	1.13
DISULFATES				
5-Androstene-3β, 17α-diol	0.48	0.48	0.53	0.53
5-Androstene-3β, 17β-diol	0.57	0.57	0.61	0.61
5-Pregnene-3β, 20α-diol	1.06	1.06	1.12 - 1.14	1.14
UNCONJUGATED				
Progesterone (O-methyl oxime trimethyl silyl ether derivative)	(1.74 (1.79	(1.74 (1.80	1.17	1.16

X) Mixture of compounds.

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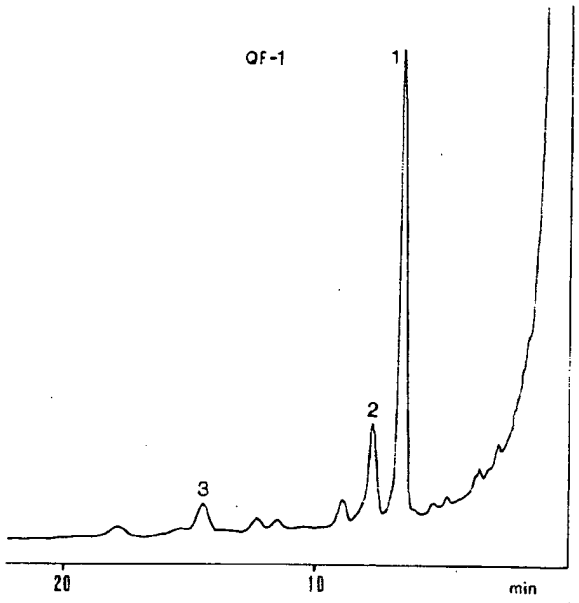

Figure 2. GLC analysis of the TMS ethers of neutral steroids in the disulfate fraction of fetal blood plasma. 3 % QF-1, 215°. The numbers refer to the TMS ether derivatives of 1 = 5-androstene-3β,17α-diol, 2 = 5-androstene-3β,17β-diol, 3 = 5-pregnene-3β,20α-diol.

The concentrations of the steroids identified were determined in blood plasma samples collected from fetuses of both sexes and from the fetal as well as placental side of the umbilical cord. In addition, from two fetuses it was possible to obtain blood samples large enough to permit their separate analysis. The results of the estimations are given in Table 2. Progesterone was the only neutral steroid found in the fraction of unconjugated steroids. The sensitivity of the method used with the plasma samples available (2.4-5 ml) did not allow the detection of compounds present in concentrations lower than 1 μg/100 ml of plasma. For C_{21} steroids with several functional groups, the limit is still higher, owing to their late elution from the GLC columns used.

Several neutral steroids were present as sulfates in fetal blood plasma (Table 1), 16α-hydroxydehydroepiandrosterone, dehydroepiandrosterone, pregnenolone and 3β,17β-dihydroxy-5-androsten-16-one being the main compounds in the monosulfate fraction and 5-androstene-3β,17α-diol in the disulfate fraction (Table 2).

Table 2. Quantitations of neutral steroids in human fetal and placental blood (μg/100 ml plasma).

Compound	Fetal blood				Placental blood	
	I[x]	II	III	IV	V	VI
MONOSULFATES						
Dehydroepiandrosterone	59	88	158	56	65	53
5-Androstene-3β,17α-diol	2	6	7	8	11	7
5-Androstene-3β,17β-diol	4	1	4	3	7	3
16α-Hydroxydehydroepiandrosterone	125	167	131	300	107	101
3β,17β-Dihydroxy-5-androsten-16-one	26	51	155	29	53	31
Pregnenolone	45	72	126	37	47	42
5-Pregnene-3β,20α-diol	17	31	18	36	47	15
3β,16α-Dihydroxy-5-pregnen-20-one	36	48	62	103	21	23
3β,17α-Dihydroxy-5-pregnen-20-one	33	43	37	32	21	15
Total monosulfates	347	507	698	604	379	300
DISULFATES						
5-Androstene-3β,17α-diol	45	85	83	110	66	48
5-Androstene-3β,17β-diol	12	26	20	25	25	19
5-Pregnene-3β,20α-diol	9	14	13	13	16	12
Total disulfates	66	125	116	148	107	79
Progesterone		22				18

x) I = from 8 fetuses (both sexes, gestational age 10-14 weeks)
II = from 8 male fetuses (gest. age 13-15 weeks)
III = from 5 female fetuses (gest. age 13-15 weeks)
IV = from one female fetus (gest. age 17 weeks)
V = from one female fetus (gest. age 20 weeks)
VI = from 7 fetuses (both sexes, gest. age 11-16 weeks)

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DISCUSSION

The method applied to the analysis of neutral steroids in fetal blood plasma was largely the same as that used in the analysis of steroid mono- and disulfates in adult human plasma (22). Combined GC-MS was used in the identification of the steroids and in the checking of the purity of their GLC peaks. In these studies it was found that a more extensive fractionation of the steroids on silicic acid was necessary to obtain specific determinations of fetal plasma steroids. In this investigation, the fraction of the unconjugated steroids separated by chromatography on Sephadex LH-20 (22) was analysed, too.

All the steroids identified as sulfate conjugates have been reported to be present in the umbilical cord blood at the end of pregnancy (7-14, 17). Comparisons of the concentrations of the sulfated steroids in the fetal blood plasma of early and mid-pregnancy with those reported in blood plasma of term fetuses (17) show that the concentrations are very similar. It seems, however, that the concentrations of the epimeric androstenediols, 5-androstene-3β,17α- and -17β-diol, increase towards the end of pregnancy (see 17). This corresponds to the observation that their concentrations in the amniotic fluid of early and mid-pregnancy are considerably lower than in term amniotic fluid (5, 6). The site of formation of these androstenediol disulfates is somewhat obscure, because only the 17β epimer, the minor compound in fetal blood plasma, has been found in low concentrations and as a monosulfate in fetal liver tissue (3). Small amounts of both compounds are present as disulfates in fetal testes (4) but the blood plasma of female fetuses contains these two steroids in amounts comparable to those present in the plasma of males (Table 2).

Blood samples were collected from both the placental and the fetal side after section of the umbilical cord, the former representing umbilical venous blood and the latter umbilical arterial blood. From the limited number of samples available it seems that the concentrations of the 16α-hydroxylated compounds, 16α--hydroxydehydroepiandrosterone and 16α-hydroxypregnenolone, as

x) I = from 8 fetuses (both sexes, gestational age 10-14 weeks)
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V = from one female fetus (gest. age 20 weeks)
VI = from 7 fetuses (both sexes, gest. age 11-16 weeks)

Progesterone

well as that of 3β,17α-dihydroxy-5-pregnen-20-one, are higher in the blood flowing to the placenta than in the blood returning from it, suggesting the removal of these compounds by the placenta (Table 2). This difference in the concentrations of 16α-hydroxydehydroepiandrosterone sulfate has already been reported in fetuses at term (11, 12) and obviously is due to the synthesis of estriol from 16α-hydroxydehydroepiandrosterone (see e. g. 1, 2).

The concentration of progesterone in cord venous blood plasma at parturition has been reported to be 24-153 μg/100 ml (mean values reported by different authors 49-102 μg/100 ml, summarized in 30). Therefore it seems (see Table 2) that in the course of pregnancy the concentration of progesterone in the fetal blood increases, as has been shown to be the case in the maternal blood (see 30). Compared with the published values for the concentration of progesterone in maternal blood at the 1st and 2nd trimester of pregnancy (see 30), the concentration of progesterone in fetal blood is about twofold. This likewise corresponds to the findings at the end of pregnancy (see 30).

It is interesting to observe that at the level of detection (about 1 μg/100 ml blood plasma) progesterone is the only unconjugated steroid in the blood flowing to and from the placenta. Therefore it seems that the steroids liberated from sulfated precursors by the active placental sulfatases (31) are rapidly converted to estrogens or secreted into the maternal compartment.

ACKNOWLEDGMENTS

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